Biochemical, Molecular, and Functional Characterization of PISCF-Allatostatin, a Regulator of Juvenile Hormone Biosynthesis in the Mosquito Aedes aegypti

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Aedes aegypti PISCF-allatostatin or allatostatin-C (Ae-AS-C) was isolated using a combination of high performance liquid chromatography and enzyme-linked immunosorbent assay (ELISA). The matrix-assisted laser desorption/ionization time-of-flight (TOF) mass spectrum of positive ELISA fractions revealed a molecular mass of 1919.0 Da, in agreement with the TOF/TOF mass spectrometry analysis of the corresponding Ae-AS-C cDNA amplified by PCR, and the sequence of the peptide was confirmed. An in vitro radiochemical assay was used to study the inhibitory effect of synthetic Ae-AS-C on juvenile hormone biosynthesis by the isolated corpora allata (CA) of adult female A. aegypti. The inhibitory action of synthetic Ae-AS-C was dose-dependent; with a maximum at $10^{-9}$ M. Ae-AS-C showed no inhibitory activity in the presence of farnesolic acid, an immediate precursor of juvenile hormone, indicating that the AE-AS-C target is located before the formation of farnesolic acid in the pathway. The sensitivity of the CA to inhibition by Ae-AS-C in the in vitro assay varied during the adult life; the CA was most sensitive during periods of low synthetic activity. In addition, the levels of Ae-AS-C in the brain were studied using ELISA and reached a maximum at 3 days after eclosion. These studies suggest that Ae-AS-C is an important regulator of CA activity in A. aegypti.

Juvenile hormone (JH)3 titers must be modulated to permit the normal progress of development and reproduction in insects (1, 2). In adult female Aedes aegypti mosquitoes, JH levels are low at adult eclosion, elevated in sugar-fed females, and low again after a blood meal (3). JH titer is fundamentally controlled by the rate of biosynthesis in the corpora allata gland (CA) (4). The rate of CA activity is, in turn, regulated, in part, by allatostatic (inhibitory) or allatotropic (stimulatory) activity (5, 6). We previously reported that the biosynthetic activity of the A. aegypti CA in vitro was inhibited by mosquito brain extracts (5), and then we used confocal, laser-scanning microscopy studies to show specific patterns of immunostaining for AS-C in the cells of the brain of A. aegypti (7).

Here we describe the purification, biochemical, molecular, and functional characterization of A. aegypti PISCF-allatostatin (or AE-AS-C). It is the first comprehensive report since the original description of a member of this peptide family as an inhibitor of JH synthesis in the lepidopteran Manduca sexta (8). In addition, we describe the developmental-stage-dependent Ae-AS-C inhibition of JH synthesis, as well as the changes in peptide levels in head samples. These studies suggest that Ae-AS-C is an important regulator of CA activity in A. aegypti.

MATERIALS AND METHODS

Chemicals—(E,E)-Farnesolic acid (FA) was purchased from Echelon (Salt Lake City, UT), and mevalonolactone, a precursor of mevalonic acid (MVA) was from Sigma.

Insects—A. aegypti of the Rockefeller strain were reared at 28 °C and 80% relative humidity with a photoperiod cycle of 16 h of light/8 h of dark as previously described (4). Adult virgin females were offered a cotton wool pad soaked in a 3% sucrose solution until 12–16 h before blood feeding. We will refer to the cotton wool pad sucrose-fed females as “sugar-fed.” Three-day-old female mosquitoes were fed pig blood equilibrated to 37 °C, and ATP was added to the blood meal to a final concentration of 1 mM immediately before use as previously described (9).

Extraction and Purification of A. aegypti Allatostatin-C—The mosquitoes were homogenized in Bennett’s solution (1% NaCl, 5% formic acid, and 1% trifluoroacetic acid in 1 M HCl) (10). After centrifugation (14,000 × g, 10 min), the
supernatant was loaded onto a Sep-Pak SPE C18 cartridge (Waters, Milford, MA), which had been equilibrated with aqueous 0.1% trifluoroacetic acid and then eluted with 80% acetonitrile in 0.1% trifluoroacetic acid. The eluate was then lyophilized. The residue was dissolved in water and subsequently stirred overnight with 200 ml of SP-Sephadex C-25 resin (Amersham Biosciences) that was previously equilibrated with 1 M acetic acid. The resin was poured into a column and washed with 1 M acetic acid. The fractions were sequentially eluted from the column with increasing concentrations of ammonium acetate (pH 7.0). The fractions were checked for immunoreactivity using the Ang-AS-C ELISA described below. Immunopositive fractions (eluted with 0.1 and 0.2 M ammonium acetate) were combined, loaded onto a Sep-Pak SPE C18 cartridge and eluted with 80% acetonitrile in 0.1% trifluoroacetic acid. The eluate was assayed to confirm immune reactivity with the Ang-AS-C ELISA, dried and analyzed by HPLC. The HPLC protocol was as follows. A Dionex Summit system (Dionex, Sunnyvale, CA) equipped with a UVD 170U detector, 680 HPLC pump, TCC 100 column oven, and Chromelone software was employed. The first column was an Ultrasphere C18 (4.6 × 250 mm) (Beckman, Fullerton, CA) eluted with a gradient of 5–60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The collected fractions were assayed for immune reactivity by ELISA as described. Positive fractions were combined and loaded onto a second Ultrasphere C18 column (4.6 × 150 mm) (Beckman) and eluted with the same solvent system and assayed for immune reactivity. Positive fractions were again combined, loaded onto a third column, a Wide-Pore C8 (4.6 × 250 mm) (J. T. Baker, Phillipsburg, NJ), and eluted with the same gradient of solvents.

Sample Preparation for Mass Spectrometry—The HPLC fraction that tested positive against A. gambiae allatostatin antiserum was reconstituted in ultrapure water (18 MΩ cm; Barnstead, Dubuque, IA) containing 0.1% trifluoroacetic acid. Ae-AS-C was then desalted and concentrated using C18 Zip Tips (Millipore, Billerica, MA) following the manufacturer’s protocols. Two µl of the desalted peptide solution were premixed with an equal volume of a 10 mg/ml α-cyano-4-hydroxycinnamic acid solution (Sigma-Aldrich). One micro liter of the mixture was deposited on a 192-well stainless steel MALDI plate and allowed to dry before analysis.

MALDI-TOF and TOF/TOF Analysis—Mass spectra were acquired in the 1–6-kDa range via tandem matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA) equipped with a diode-pumped Nd:YAG laser (200 Hz, 355 nm). Analyses were performed in delayed extraction positive ion reflectron mode at an accelerating voltage of 20 kV, with the data system operating under automatic control. Tandem mass spectra (MS/MS) were obtained with a 1-Da precursor ion selection window, and ion activation was achieved using high energy collision-induced dissociation. Smoothed and denoised mass spectra were exported in ASCII format from the Data Explorer MS software and input into Excel. Experimental fragment ion peak lists were compared with theoretical peak lists for the peptide sequence deduced from the cDNA. A match was considered positive if the difference in mass values was less than 0.1 Da. Theoretical fragment ion peak lists were obtained using the MS product on-line application. A user-defined amino acid with the elemental formula CnH2nONS was included in the query to represent the two oxidized cysteine residues.

Bioinformatic Analysis—The known motif QIRYRQCYP—PISC—was used to query a custom nucleotide data base created from the public available contigs of A. aegypti via WU-blast software. To compensate for the small size of the query sequence the following parameters were used: W = 3 (word size), T = 14 (neighborhood word threshold), and E = 10 (default value; the E from the Karlin-Altshul equation). All of the consecutive BLAST analyses were done using the NCBI BLAST suite of programs with the default parameters. Multiple alignments and phylogenetic trees were calculated with the clustalx software (11).

RNA Extraction and cDNA Cloning—Total RNA was isolated from adult female mosquitoes using RNA-binding glass powder as previously described (12). Contaminating genomic DNA was removed with Optizyme™ RNase-free DNase I (Fisher). First Strand cDNA synthesis was carried out using SuperScript® II first strand synthesis system for reverse transcription-PCR (Invitrogen). PCR was performed in a Mastercycler gradient (Eppendorf, Westbury, NY) using Taq DNA polymerase (Promega, Madison, WI). PCR products were cleaned with the QIAquick PCR purification kit (Qiagen) and sequenced by the DNA Core Sequencing Facility of Florida International University (Miami, FL).

Synthetic A. aegypti Allatostatin—Ae-AS-C was provided by Alpha Diagnostic International (San Antonio, TX), purified by reversed phase liquid chromatography, and assessed to be ≥99% pure by analytical reversed phase liquid chromatography, MALDI-TOF MS, and amino acid analysis.

Stock aqueous solutions of synthetic allatostatin (10⁻³ M) were stored in aliquots at −80°C. For each assay, a new aliquot was removed from the stock and dried under nitrogen, and dilutions were made by adding the required amount of incubation medium.

In Vitro Radiochemical Assay for CA Activity—The CA complexes were isolated as previously described (4). Rates of JH biosynthesis were estimated by the in vitro radiochemical assay (13, 14), as previously modified (4, 6). Briefly, CA complexes were incubated for 4 h in fresh medium containing 3H-labeled methionine. Under our assay conditions, the incorporation of 3H-labeled methionine into JH III was linear for at least 6 h. After extraction and separation by thin layer chromatography, the JH III band was removed, placed into scintillation mixture, and assayed for 3H. The quantity of JH produced was calculated from the specific activity of the 3H-labeled methionine in the medium. The effect of Ae-AS-C, FA, and MVA were tested by adding them directly into the CA incubation medium.

Primary Antibodies against Anopheles gambiae Allatostatin—Polyclonal antisera were custom prepared by Genemed Synthesis Inc. (San Francisco, CA) using rabbits injected with Ang-AS-C conjugated to keyhole limpet hemocyanin as previously described (7).

Competitive ELISA for PISC—Allatostatin—Mosquito heads were collected by decapitation. The samples were homogenized
in 80% acetonitrile and centrifuged at 14,000 × g for 10 min at 4 °C. The supernatants were recovered and stored at −80 °C. Standard curves were produced using synthetic Ang-AS-C made by Biopeptide Company (San Diego, CA).

Two 96-well plates were used for each assay. For Plate 1, 2000 fmol of conjugated keyhole limpet hemocyanin-conjugated Ang-AS-C (dissolved in 20 μl of 80% acetonitrile) were coated onto wells by drying at 37 °C for 90 min and then incubated overnight at 4 °C with 100 μl of a 0.1 M sodium carbonate/sodium bicarbonate buffer (coating buffer, pH 9.6). After washing three times with 100 μl of 0.1M phosphate buffer, pH 7.4, 0.1% Tween 20 (PBS-T), 150 μl of blocking solution were added to each well (1% bovine serum albumin in PBS), and the plate was incubated for another 90 min at 37 °C.

For Plate 2, head samples or standards (10–160 fmol of Ang-AS-C) in 20 μl of 80% acetonitrile were dried in the wells at 37 °C for 90 min and then incubated at 4 °C with 100 μl of a 0.1 M sodium carbonate/sodium bicarbonate buffer (coating buffer, pH 9.6). After washing three times with 100 μl of 10 mM phosphate buffer, pH 7.4, 0.1% Tween 20 (PBS-T), 150 μl of blocking solution were added to each well (1% bovine serum albumin in PBS), and the plate was incubated for another 90 min at 37 °C.

Plate 1 was incubated for 90 min at 37 °C. After washing three times with 100 μl of PBS-T, 100 μl of secondary antibody (goat anti-rabbit monoclonal antibody conjugated to horseradish peroxidase, 1:3000 in blocking solution) were added to each well. The plate was then incubated for 60 min at 37 °C. After three final PBS-T washes, 100 μl of substrate mixture of tetramethyl benzidine and H₂O₂ (Pierce) were added to each well and incubated for 20 min at room temperature. The reaction was stopped by the addition of 100 μl of 2 M H₂SO₄ to each well, and optical density was read at 450 nm on an Elx 808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT).

Statistical Analysis—Statistical analyses of the data were performed by t test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). The results were expressed as the means ± S.E. and considered significantly different at p < 0.05.

RESULTS

Isolation and Biochemical Characterization of Ae-AS-C—A single positive immunoreactive HPLC fraction was isolated and lyophilized, and its primary structure and molecular mass were obtained by MALDI-TOF (Fig. 1) and MALDI-TOF/TOF MS (Fig. 2). The MALDI-TOF spectrum reveals a protonated molecular ion ([M+H]+) at m/z 1919.0, in agreement with the expected value of m/z 1918.9 for qIYRQCFYPISCF with the two cysteines bridged, and where “q” stands for a pyroglutamic acid residue. High energy collision-induced dissociation on the 1919.0 precursor ion produced diagnostic fragments that confirmed the proposed amino acid sequence (Fig. 2). Because of the cyclic nature of the C-terminal portion of this peptide, a complete series of ladder y and b ions was not obtained. However, internal and immonium fragment ions originated within the cyclic peptide domain confirmed the proposed sequence.

Molecular Cloning of Ae-AS-C—The A. aegypti genome available at NCBI was searched for sequences that were an exact match to the QIYRQCFYPISCF peptide, resulting in the alignment with contig 1047197605538 (accession number AAGE0111121).

We consequently designed primers against specific regions of this gene and carried out PCR using A. aegypti cDNA as template. This resulted in the amplification of the complete cDNA coding for the allatostatin-C preprohormone (cited as A. aegypti experimental in Fig. 3A). The Ae-As-C sequence was used to query again the nonredundant data base at NCBI via position iterated BLAST (PSI BLAST) to reveal similar sequences. After 10 iterations, sequences from Drosophila pseudoobscura, (GA13352-PA), Drosophila melanogaster (AF346433_1), Samia cynthia ricini (AA683691_1), Pseudoaletia unipuncta (AA9A327_1), and Spodoptera frugiperda (Q868F8) were obtained with alignments above threshold (Fig. 3A).

ENSEMBL pipeline revealed a possible candidate for a full-length Aedes allatostatin (transcript 25005.m03793 in TIGR/VB genome
PISCF-Allatostatin and JH Synthesis in Mosquitoes

In *A. gambiae* two different ortholog peptides have been predicted by the ENSEMBL pipeline. Both consist of two exons that show high similarity with exons 2 and 3 of the Ae-AS-C (75 and 83.8% identity, respectively). The manually submitted sequence from M. Brown (15) is longer and supports the existence of a third exon, similar to exon 1 of *A. aegypti* (75.9% identity). Furthermore, the existence of this third exon is supported by expressed sequence tags that cover the region and by SNAP predictions (supplemental Fig. SII).

In *D. melanogaster* the ortholog AS-C is being mapped into a region filled with five genes. Fewer genes are observed in *A. gambiae* and *A. aegypti* that might simply be reflecting the intensity of studying of *D. melanogaster*. The coding sequence consisted of three exons, although the 5′-untranslated region contains an additional exon/intron donor site (outline boxes in supplemental Fig. SII). It is very interesting that in the same strand, in a distance of less than 100 Kbp from AS-C, is located the gene Samuel (CG31868). The potential orthologs of Samuel (unique best reciprocal hits) in *A. aegypti* (AAEL005643) and *A. gambiae* (ENSANGT00000012793) are located at the same relative distance from AS-C but in the opposite strand (supplemental Fig. SII).

**Effect of Ae-AS-C on JH Biosynthesis**—The effect on JH synthesis of increasing concentrations of Ae-AS-C (10⁻¹⁰–10⁻⁶ M) was tested on glands with high activity (3-day-old sugar-fed females) and low activity (females 24 h after a blood meal). Maximal inhibition of JH biosynthesis in both sugar-fed and blood-fed females occurred at a concentration of 10⁻⁶ M (results not shown); this concentration was used in all subsequent experiments.

In sugar-fed females, acquisition of sensitivity to a low concentration of AS-C occurs in the CA connected to the brain 3 days after emergence (Fig. 4A). Similar results were obtained in sugar-fed females when CA were disconnected from the brain (results not shown).

In blood-fed females, CA disconnected from the brain are very sensitive to AS-C during the first 24 h (Fig. 4B). Similar results were obtained in blood-fed females when CA were connected to the brain (results not shown).

The addition of exogenous FA stimulates JH synthesis in a dose-response fashion (6). We analyzed the effect of Ae-AS-C on the rate of JH biosynthesis by FA-stimulated CA connected to the brain. Ae-AS-C showed no inhibitory activity on FA-stimulated CA connected to the brain from either 3-day-old sugar-fed females or females 24 h after blood feeding (Fig. 5).

Based on contig 9205, the Ae-AS-C cDNA has the following genomic structure: Exon 1 complement (23464–23571), Exon 2 complement (1828–1961), and Exon 3 complement (1001–1112) (supplemental Fig. SIIA). Intron 1 is 21 Kb in length and is littered with repeats including tandem repeats, transposable elements, etc. (all coordinates mentioned in reference to contig 9205). A complete annotation of the repeats in contig 9205 is available through VectorBase.
FIGURE 3. Peptide comparison. A, multiple alignments of A5-C peptides found in GenBank™, ENSEMBL, and VectorBase. *Ae_aegypti_exp.* is the sequence we reported during this study, *Ae_aegypti_predicted* is the allatostatin previously predicted by ENSEMBL (AAEL005747). More details for each sequence can be found under “Material and Methods.” B, unrooted tree based on the peptide alignment showed in A. The same naming scheme is used. *S. frugiperda* and *P. unipuncta* are Lepidoptera Noctuoidea. *S. cynthia ricini* belongs to Lepidoptera Bombyoidea. *D. melanogaster* and *D. pseudoobscura* are Diptera Brachycera, and *A. gambiae* and *A. aegypti* are Diptera Nematocera.
Similar results were obtained in blood-fed females when CA were disconnected from the brain (results not shown).

The addition of exogenous MVA, an early precursor of JH (16, 17), stimulated the synthesis of JH by CA (Fig. 6A). Maximal stimulation of JH biosynthesis occurred at a concentration of 160 µM; therefore, this concentration was used in all subsequent experiments. Ae-AS-C reduced JH biosynthesis by MVA-stimulated CA in both sugar-fed females and blood-fed females (Fig. 6, B and C).

Ae-AS-C Contents in Heads—The competitive ELISA demonstrated a linear relationship and allowed detection of Ae-AS-C at the fmol level. The quantity of AS-C in heads was determined in sugar-fed A. aegypti females; levels of Ae-AS-C were below 2 fmol in newly eclosed females, increased to a maximum of 40 fmol by 3 days after emergence, and decreased after that to a level of 6 fmol a week after emergence (Fig. 7).

**DISCUSSION**

Classical endocrinological studies have shown that the insect CA is subject to regulation from the brain (18). Factors from the brain also modulate CA activity in mosquitoes; a significant reduction of JH synthesis is observed when CA are incubated with isolated brains, in medium in which brains have been maintained (preconditioned medium), or in medium with brain extract, suggesting that allatostatin-like factors are present in the mosquito brain (5).
Insect allatostatins can be grouped into three families: YXFGL-amide-allatostatins (cockroach or type A) (AS-A), \( W_2 W_4 \)-allatostatins (cricket or type B), and PISCF-allatostatins (type C) (AS-C) (19, 20). In recent work, we have shown that none of the five \( A. aegypti \) allatostatin-A had an effect on JH synthesis in female mosquito; on the other hand AS-C from \( A. gambiae \) showed a significant inhibitory response (5). In addition, confocal laser-scanning microscopy studies using antisera against \( A. gambiae \) AS-C revealed intense immunostaining for four cells in each protocerebral lobe in the brain of adult female \( A. aegypti \) and \( A. albimanaus \) (7). These findings prompted us to hypothesize that an AS-C homolog could be the brain factor involved in regulation of JH biosynthesis in \( A. aegypti \) females. Therefore, using a combination of HPLC and competitive-ELISA, we isolated and characterized an AS-C homolog from \( A. aegypti \) females. The sequence of this peptide proved to be identical to the \( A. gambiae \) PISCF-allatostatin (15). The allatotropin from \( A. aegypti \) and \( A. gambiae \) are also identical (15, 21), and the five AS-A are very similar in these two species (15, 22), confirming the high degree of conservation between regulatory peptides in members of two mosquito subfamilies that diverged more than 100 million years ago (23).

Allatostatin-C was originally described as a JH synthesis inhibitor in the lepidopteran \( M. sexta \) (8). The \( Aedes \) allatostatin-C first described here shares the common characteristics of all PISCF-allatostatins identified up to date in insects, which are 15 amino acids long, with a disulfide bridge between \( C_7 \) and \( C_{14} \). \( M. sexta \) and \( Drosophila \) AS-Cs differ from each other by a single amino acid substitution: Phe for Tyr at position 4. The two mosquito peptides have an additional amino acid substitution: Ile for Val at position 2.

The tree constructed based on the allatostatin peptides revealed by PSI-BLAST (Fig. 3B) clearly separates not only Lepidoptera from Diptera but also within those taxa Noctuoidea from Bombycoidea in the case of Lepidoptera and Brachycera from Nematocera in the case of Diptera. However, only the sequence of the mature peptide is significantly conserved among all the AS-C genes described in this study (Fig. 3A).

The gene structure of allatostatin C is similar in \( D. melanogaster \), \( A. gambiae \), and \( A. aegypti \). Single genes containing three different exons are responsible for the final preprohormone. Despite the prediction of a two exon gene in \( A. gambiae \), the expressed sequence tags available, the manual annotation, the SNAP predictions, and the BLAST comparison with \( A. aegypti \) clearly support a three-exon gene model (supplemental Fig. SII). The fact that the two \( A. gambiae \) AST2 genes predicted by ENSEMBL are identical (supplemental Fig. S1) leads to the hypothesis that the failure of the automatic pipeline to predict correctly the gene structure is either due to an assembly-related error or due to the fact that a very recent duplication occurred in \( A. gambiae \) covering only part of the AST2 gene. The genome organization in the region, and most notably the presence of another \( D. melanogaster \) gene (CG31868) and its potential orthologs in similar distances, might be indicative of a microsynteny between these three species (supplemental Fig. SII).

Very little information is available on the structural requirements of AS-C for biological activity. Reduced disulfide bridge, alanine substitution, and N-terminal deletion have been used to analyze the structure-activity relationship of \( M. sexta \) AS-C on JH biosynthesis and contractions of the foregut of larval lepidoptera \( Lacanobia oleracea \) (24, 25); breaking the disulfide bond between \( C_7 \) and \( C_{14} \) drastically reduced the biological activity. The presence of the \( C_7 \) and \( C_{14} \) disulfide bond is also essential for high affinity binding of \( D. melanogaster \) AS-C to the \( Drosophila \) AS-C receptor (26). On the other hand \( M. sexta \) AS-C loses potency but remains fully active if the first five N-terminal amino acids are deleted, confirming that the “active cores” of many insect neuropeptides, necessary to activate the relevant receptor, reside in the C terminus of the molecule. The two amino acid substitutions described in mosquitoes are located within the first 5 N-terminal residues, corroborating that changes in this part of the molecule do not extensively affect the biological activity.

\( Ae-AS-C \) modulates CA activity in \( A. aegypti \) females. There were significant reductions of JH biosynthesis rates when CA complexes from females, both before and after blood feeding, were incubated with \( 10^{-9} \text{ M } Ae-AS-C \).

There are physiological conditions when the CA is partially or completely refractory to regulation by allatostatins (27). We observed that the sensitivity of the isolated CA to \( Ae-AS-C \) changed during development. The CA was more sensitive at times when the levels of JH were decreasing, such as 3 days after emergence in sugar-fed females or during the first 24 h after blood feeding. These decreases in CA sensitivity to allatolegulatory peptides could be the result of lower titers of allatal receptors or to reductions in the efficiency of signal transduction mechanisms connected to key enzymes in the pathway. Similar changes in sensitivity were observed for the effect of allatotropin on the CA of \( A. aegypti \) females (6).

The biochemical targets in the CA subject to AS regulation are yet unidentified, although for AS-A, they usually appear to be located early in the sesquiterpenoid pathway (29). Sutherland et al. (29) found that cockroach AS is more effective as an inhibitor of JH synthesis in glucose- or amino acid-driven glands than in acetate-driven glands; they proposed that inhibition of JH biosynthesis by the cockroach AS-A occurs at the first committed step(s) of JH synthesis, i.e. the transfer of 2C units from mitochondria to the cytoplasm by the tricarboxylate
carrier and/or the ATP-citrate lyase. Inhibition of JH biosynthesis in cockroach by AS is relieved by farnesol and by mevalonate, and neither HMG-CoA reductase nor HMG-CoA synthase (HMG-CoA synthase and acetoacetyl-CoA lyase) are inhibited by AS-A (29), indicating that the target for allatostatin is located before the entrance of these molecules into the synthetic pathway. However, in this study, we found that inhibition of JH biosynthesis in A. aegypti by AS-C is relieved by the addition of FA and not by MVA. A possible explanation is that AS-C partially inhibited target enzyme(s) between MVA and FA formation, although the use of exogenous mevalonate as a probe of the physiology of the CA is made difficult by the limited penetration of MVA into the gland cells (28).

We detected significant changes in the amount of Ae-AS-C in the heads of sugar-fed female mosquitoes, showing a maximum at 3 days after eclosion. These changes may be related to physiological requirements of the CA, and although the concentration of peptide in the brain itself does not reveal the amount that is released in the axon terminals of the CA, the highest value correlates with an increase in the sensitivity of the CA to inhibition by Ae-AS-C, as well as with a decrease in the CA synthetic activity.

In summary, these studies demonstrate that A. aegypti PISCF-allatostatin is a true allatostatin, which shows a dose-response relationship and age-specific effects on JH synthesis. This is the first isolation and identification of an AS-C mature peptide in Diptera, as well as the first comprehensive description of an allatostatic effect of PISCF-allatostatins outside the Lepidoptera, suggesting that members of the PISCF-allatostatin family might play an important role in the regulation of JH synthesis in holometabolous insects.

Acknowledgment—We thank Marcela Nouzova for helpful comments on the manuscript.

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