ELSEVIER

Inhibition of juvenile hormone biosynthesis in mosquitoes: effect of allatostatic head factors, PISCF- and YXFGL-amide-allatostatins

Yiping Li[a], Salvador Hernandez-Martinez[b], Fernando G. Noriega[a],*

[a] Department of Biological Sciences, Florida International University, Miami, FL 33199, USA
[b] Centro de Investigaciones Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Cuernavaca, Morelos, 62508100, Mexico

Abstract

We investigated the role of head factors and allatostatins (ASs) on the regulation of juvenile hormone (JH) synthesis in female adult mosquito. The biosynthetic activity of the *Aedes aegypti* corpora allata (CA) in vitro was inhibited by factors present in the head. Disconnecting the CA from the brain resulted in a significant increase in the rate of JH biosynthesis. Inhibition was not dependent on intact nervous connections; co-incubation of CA with brains or brain extracts resulted in a significant decrease of JH biosynthesis. This inhibitory effect of brain extracts was reversible and heat stable; extracts lost the inhibitory activity after proteinase K digestion suggesting a peptidic structure. In a first attempt to elucidate the nature of this inhibitory factor, we tested in our CA in vitro system the effect of members of two families of allatostatins already described in mosquitoes. *Anopheles gambiae* PISCF-allatostatin (homolog to Manduca PISCF-allatostatin) significantly inhibited JH synthesis, while *Ae. aegypti* YXFGL-amide-allatostatins (homologs to cockroach YXFGL-amide-allatostatins) did not affect JH synthesis. These results represent the first description of an allatostatic effect of PISCF-allatostatins outside the Lepidoptera.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Aedes aegypti; Mosquito; Juvenile hormone; Corpora allata; Allatostatin

1. Introduction

Juvenile hormones (JHs) are a class of regulatory sesquiterpenoids that control metamorphosis in immature insects and reproduction in adult insects. The corpora allata (CA), a pair of endocrine glands with nervous connections to the brain, synthesize and release JH. The regulation of JH synthesis is achieved by a complex interplay of stimulatory and inhibitory factors, such as neuropeptides, JH precursors, biogenic amines and sex peptides [1,2]. A large number of structurally diverse neuropeptides that stimulate or inhibit JH biosynthesis, termed allatotropins (ATs) and allatostatins (ASs), respectively, have been isolated from different insect species [3–7]. To date, three families of allatostatins have been identified in insects: cockroach allatostatins (YXFGL-amide or type-A), cricket allatostatins

$(W_2W_9$ or type-B) and Manduca allatostatins (PISCF or type-C) [1,8].

JH levels in the yellow fever mosquito *Aedes aegypti* increase during the first 2 days after adult emergence and remain high in sugar-fed females [9]. When a female takes a blood meal, the JH levels fall rapidly and reach its lowest point 24 h after the blood meal [9]. These changes in JH levels are primarily the result of concomitant changes in CA activity. Rates of JH biosynthesis closely reflect the levels of JH in the sugar-fed mosquito and after a blood meal; JH biosynthesis by the isolated CA of *Ae. aegypti* females is high in sugar-fed females and significantly decreases after blood feeding [10].

Allatostatins might be partially responsible for the decrease of CA activity after blood feeding. In these studies, a bioassay was developed to test the existence of allatostatic factor(s) in the head of *Ae. aegypti*. Our results show that factor(s) present in the head inhibits JH biosynthesis by the isolated CA. This heat-stable factor is released into the medium. In addition, we tested the effect on JH synthesis of two families of allatostatins that are found in mosquitoes: YXFGL-amide-allatostatins [11] and PISCF-allatostatins [12].

---

* Corresponding author. Department of Biological Sciences, OE 167, Florida International University, 11200 SW 8TH ST Miami, FL 33199, USA. Tel.: +1-305-348-6632; fax: +1-305-348-1986.
 E-mail address: noriegaf@fiu.edu (F.G. Noriega).

2. Materials and methods

2.1. Chemicals

(*E,E*) Farnesoic acid (FA) was purchased from Echelon (Salt Lake City, UT, USA). JH III was from ICN (Irvine, CA, USA). HPLC grade ethyl acetate, hexane, methanol and C18 SEP-PAK cartridge were from Burdick and Jackson (Muskegon, MI, USA).

2.2. Insects

Ae. aegypti of the Rockefeller strain were reared at 28 °C and 80% relative humidity under a photoperiod of 16 h light/ 8 h dark. Virgin adult females were offered a cotton wool pad soaked in a 3% sucrose solution until 12–16 h before blood feeding. In this study, only virgin females were used. We 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 1 mM ATP was added to the blood meal immediately before use as previously described [13].

2.3. Dissections of corpora allata complexes

For preparation of isolated CA complexes, mosquitoes were immobilized by brief exposure to ice. To facilitate dissections, legs, wings and antennae were rapidly cut off, and the anterior half of the body was pinned to a silicon dissecting dish and covered with a drop of mosquito saline buffer (138 mM NaCl, 8.4 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 12 mM NaH_2PO_4 and 42.5 mM sucrose). The thorax was split open and the CA plus corpora cardiaca (CC) complex attached to the aorta were exposed by carefully removing the thoracic muscles, cuticle and other tissues from the neck region using a razor-blade scalpel, fine forceps and scissors. The aorta and CA–CC complex, connected to the brain and head capsule, were isolated. In most of the experiments described in this paper, we used CA + CC + aorta + brain + head capsule preparations that we will refer as "Br–CA–CC complexes" or Br–CA–CC. The dissection of one Br–CA–CC complex was completed in about 5 min. Denervated CA–CC complexes were prepared as Br–CA–CC, but CA–CC were cut off from the head and isolated attached to a small piece of aorta. Dissected tissues were rinsed in methionine-free M-199 medium and held in fresh M-199 medium without methionine for 1–2 h to consume intra-glandular methionine before transferring them to the assay medium.

2.4. In vitro radiochemical assay for CA activity

Rates of JH biosynthesis were estimated by the in vitro radiochemical assay as described by Feyereisen and Tobe [14] and Feyereisen [15], and modified by Li et al. [7,10]. Glands from a single mosquito were incubated in 100 μl M-199 assay medium with labeled L-[methyl-^{3}H] methionine (specific activity 2.96–3.11 TBq/mmol; 80–84 Ci/mmol,

Amersham Pharmacia, IL, USA). The final concentration of methionine in the medium was 50 μM and the specific activity was 0.56 TBq/mmol (15 Ci/mmol). Under these conditions, the incorporation of L-[methyl-^{3}H]-methionine into JH III was linear for at least 6 h [10]. At the end of the experimental period, incubations were terminated by the addition of 100 μl 1% EDTA, and 100 μl methanol containing 25 μg cold JH III as carrier and internal standard. The incubation medium and the gland were extracted together with 1 ml of hexane and separated by thin-layer chromatography (TLC). After TLC separation (developed in 2:1 (v/v) hexane and ethyl acetate), the JH III band was detected under UV light, cut, put into 10 ml scintillation cocktail overnight and assayed for ^{3}H. The quantities of JH produced in the experiment were calculated from the specific activity of the L-[methyl-^{3}H] methionine in the medium with assumption of a specific incorporation ratio of 1 (nonisotopic dilution). JH degradation by esterases was checked by incubating [^{3}H]-JH III in medium in the presence or absence of CA complexes and brain extracts, and analyzing the recovery of labeled JH. Between 95% and 99% of the hormone was recovered intact after 4 h of incubation (results not shown). In some experiments, the JH esterase inhibitor OTFP [16] was added to the incubation medium. Results of incubations in the presence or absence of OTFP were not significantly different (results not shown).

2.5. In vitro co-incubation of the CA with isolated brain

Brains were dissected in ice-cold mosquito saline buffer, and the attached subesophageal ganglia were removed. Brains were rinsed in M-199 medium without methionine to remove traces of hemolymph, and held in the tissue culture medium M-199 without methionine. Single Br–CA–CC complexes or CA–CC complexes were co-incubated with one brain in 100 μl fresh medium M-199 containing labeled L-[methyl-^{3}H] methionine. Controls were Br–CA–CC or CA–CC complexes incubated in the absence of brains.

2.6. Collection of preconditioned medium and incubation of CA

Ten brains without subesophageal ganglia were dissected from females 24 h after blood feeding. Brains were rinsed in M-199 without methionine and incubated in M-199 at 30 °C for 4 h under continuous gentle agitation. After incubation, tissues were removed by centrifugation ($5000 \times g$, 30 min), and the supernatants were stored at -20 °C until bioassayed. An aliquot of 10 μl of supernatant, containing 1 brain equivalent, was mixed with fresh M-199 medium to make 100 μl of the assay medium.

2.7. Brain extract preparations

Brains were dissected from blood-fed *Ae. aegypti* females in ice-cold mosquito saline buffer. Brains were homogenized

in ice-cold extraction medium—methanol/water/acetic acid, 100:10:1 (v/v/v)—in a microcentrifuge tube with a hand-held pestle. Homogenates were centrifuged at $12,000 \times g$ for 10 min. Supernatants were removed and pellets were re-extracted with medium. Supernatants were combined, frozen and dried using a Speed Vac. The lyophilized residues were dissolved in 100 μl of H_2O with 0.1% TFA and loaded into a SEP-PAK C18 (Burdick and Jackson, Honeywell, MI, USA), which was previously washed with 2 ml of methanol and equilibrated with 1 ml of H_2O with 0.1% TFA. Samples were loaded on a SEP-PAK C18 cartridge, washed with 2 ml of H_2O with 0.1% TFA and eluted with 1.2 ml of 80% methanol, 20% H_2O, 0.1% TFA. Eluted fractions were dried using a Speed Vac and stored at -80 °C until used.

2.8. Heat and proteinase K treatments of brain extracts

Brain extracts were dissolved in M-199 medium without methionine and incubated with 1 mg/ml of proteinase K at 37 °C for 4 h. For heat treatment, extracts were heated at 100 °C for 10 min in boiling water.

2.9. Reversibility of the effect of inhibitory factor(s)

Single Br−CA−CC complexes from 3-day-old sugar-fed females were subjected to two consecutive 2-h incubation periods. In the initial 2-h period, CA complexes were incubated in control medium containing labeled L-[methyl-^{3}H] methionine, or in medium containing labeled L-[methyl-^{3}H] methionine plus extracts from brains dissected from 48 h blood-fed females. After 2 h, the glands were rinsed in fresh culture medium M-199 without methionine for 30 min, and then incubated for a second 2-h period in fresh medium M-199 containing labeled L-[methyl-^{3}H] methionine. The medium from the initial incubation and the second incubation were analyzed separately as described above.

2.10. Effect of allatostatins

Ae. aegypti YXFGL-amide-allatostatins 1, 2, 3, 4 and 5 (Aea-AS 1−5) [11] were custom-synthesized at the Center for Biotechnology Research, Kansas State University (Manhattan, KS, USA), purified by reversed phase liquid chromatography and assessed to be $\geq 97\%$ pure by analytical reversed phase liquid chromatography, mass spectroscopy and amino acid analysis. *Anopheles gambiae* PISCF-allatostatin (Ang-AS) [12] was custom-synthesized by Alpha Diagnostic International (San Antonio, TX, USA) purified by reversed phase liquid chromatography and assessed to be $\geq 100\%$ pure by analytical reversed phase liquid chromatography, mass spectroscopy and amino acid analysis.

Stock aqueous solutions of synthetic allatostatins (10^{-3} M) were stored in aliquots at -80 °C. For each assay, a new aliquot was removed from the stock, dried under nitrogen, and each allatostatin solution to be tested was made by adding the required amount of incubation medium.

The final concentration of Aea-AS 1−5 in the medium was 10^{-8} or 10^{-6} M. Ang-AS was used at 10^{-9} M. In experiments with FA plus Aea-AS, 4×10^{-5} M FA and 10^{-8} M Aea-ASs were added into the medium. *Ae. aegypti* allatotropin [17] was synthesized and prepared as previously described [7].

2.11. Statistical analysis

Statistical analysis of the data was performed by *t* test or one-way analysis of variance (ANOVA) with Tukey's post-test using GraphPad Prism version 3.00 for Windows, Graph-Pad Software (San Diego, CA, USA). The results were expressed as mean ± S.E.M., and considered significantly different at $P < 0.05$. Values of percent inhibition of JH
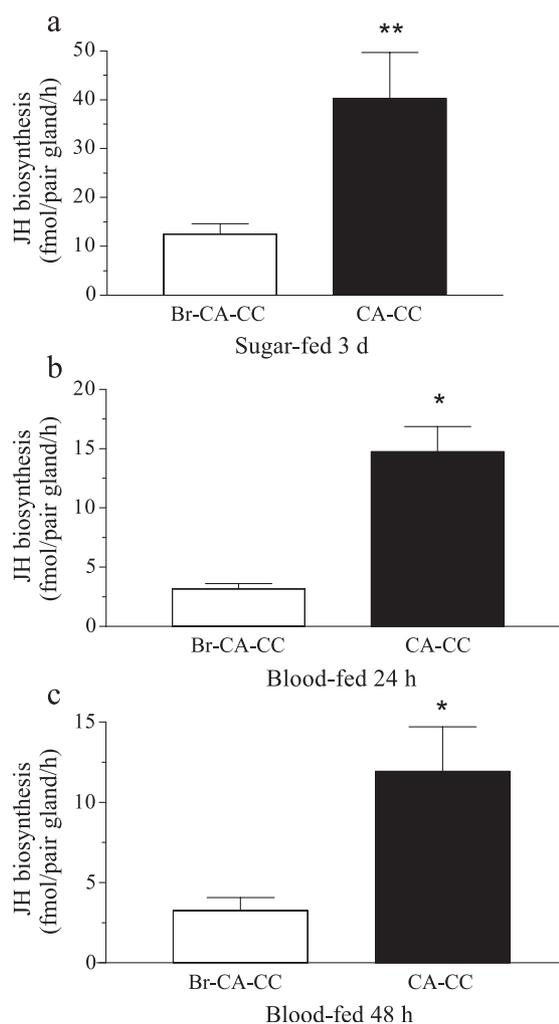

Fig. 1. Effect of denervation on JH biosynthesis. (a) Tissues dissected from 3-day-old sugar-fed females. (b) Tissues dissected from females 24 h after blood feeding. (c) Tissues dissected from females 48 h after blood feeding. Br−CA−CC: CA−CC complex attached to the brain. CA−CC: CA−CC complex disconnected from the brain. Each bar represents the mean ± S.E.M. of 5−10 independent determinations of individual CA complexes. Asterisk above each bar indicates the value is significantly higher than the Br−CA−CC in respective group by unpaired *t* test at $P < 0.05$ (*) or $P < 0.01$ (**).

biosynthesis were calculated using the following formula: percent inhibition (%) = 100 × (JH biosynthesis activity in the presence of extract − JH biosynthesis activity in the absence of extract)/JH biosynthesis activity in the absence of extract).

3. Results

3.1. Denervation increases synthesis of JH

In sugar-fed and blood-fed females disconnecting the CA−CC complex from the brain + head capsule (denervation) resulted in a significant increase in the rate of JH biosynthesis when compared with corpora allata−corpora cardiaca complexes connected to the head (Br−CA−CC) (Fig. 1). Co-incubations of the CA−CC complexes with brains isolated from females 24 h after blood feeding resulted in a significant decrease of JH biosynthesis (Fig. 2).
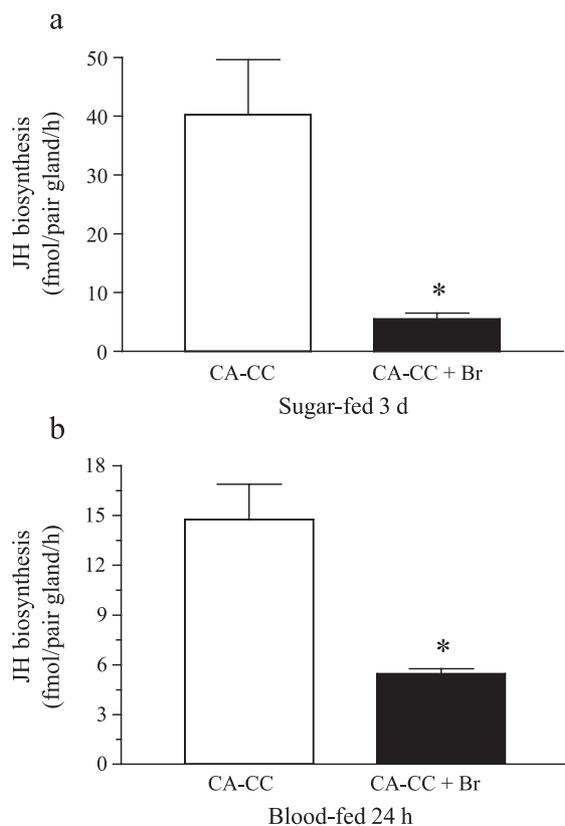
Fig. 2. Effect of co-incubation of CA with isolated brains. (a) Tissues dissected from 3-day-old sugar-fed females. (b) Tissues dissected from females 24 h after blood feeding. CA−CC: CA−CC complex disconnected from the brain. CA−CC + Br: CA−CC co-incubated with one brain dissected from females 24 h after blood feeding. Each data point represents the mean ± S.E.M. of five independent determinations of individual CA complexes. Asterisk indicates significant differences by unpaired t test at $P < 0.05$ (*) between control (no brain added) and tissue co-incubated with a brain.
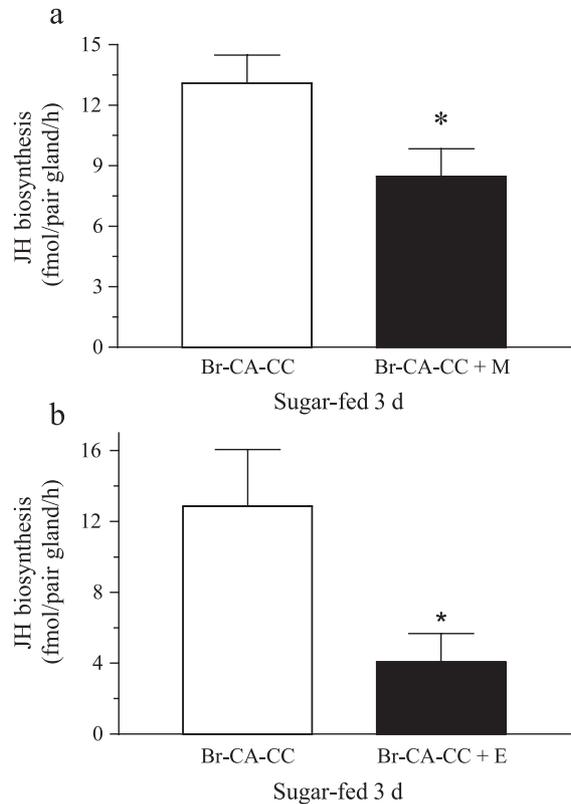
Fig. 3. Effect of co-incubation of CA complexes with pre-conditioned medium or brain extracts. (a) Br−CA−CC + M: Incubation of Br−CA−CC from 3-day-old sugar-fed females in medium pre-conditioned by incubation for 4 h with brains dissected from females 24 h after blood feeding. Each Br−CA−CC was incubated with one brain equivalent. (b) Br−CA−CC + E: Incubation of Br−CA−CC from 3-day-old sugar-fed females with methanolic extracts from brains dissected from females 48 h after blood feeding. Each Br−CA−CC was incubated with extracts from 10 brains. Br−CA−CC: control. Each data point represents the mean ± S.E.M. of five independent determinations of individual CA complexes. Asterisk indicates significant differences between control and treatments in respective group by unpaired t test at $P < 0.05$ (*).

3.2. Incubation of CA complexes with pre-conditioned medium or crude brain extracts causes inhibition of JH biosynthesis

When a single brain from a female 24 h after a blood meal was incubated in medium for 4 h, and Br−CA−CC from 3-day-old sugar-fed female subsequently incubated in this "preconditioned" medium, a significant inhibition of JH biosynthesis was observed (Fig. 3a). JH biosynthesis was significantly decreased when Br−CA−CC from 3-day-old sugar-fed females were incubated in the presence of the lyophilized extracts from 10 brains dissected from females 48 h after blood feeding (Fig. 3b).

3.3. The inhibitory activity in brain extracts changes after a blood meal

Br−CA−CC from 3-day-old sugar-fed females were used for a more detailed study of the effects of extracts from brains

dissected at different times after blood feeding. Extracts from brains dissected 24 and 48 h after feeding possessed the highest inhibitory activity (Fig. 4a). Brains showed highest inhibitory activities at the same times that the CA had the lowest biosynthetic rates (reproduced from Li et al. [10]) (Fig. 4b).

3.4. The inhibitory effect of brain extracts is reversible

To determine if the inhibitory effect of the brain factor was reversible, Br–CA–CC complexes from 3-day-old sugar-fed females were incubated for a 2-h period, with or without brain extract from 48-h-old blood-fed females (10 brain equivalents). During this first 2-h period, JH release from the CA was significantly inhibited in the presence of brain extract (Fig. 5a). Both experimental and control CA were then incubated in fresh medium without methionine for 30 min to wash out the extract, and then incubated for a second 2 h in medium without extract. No significant
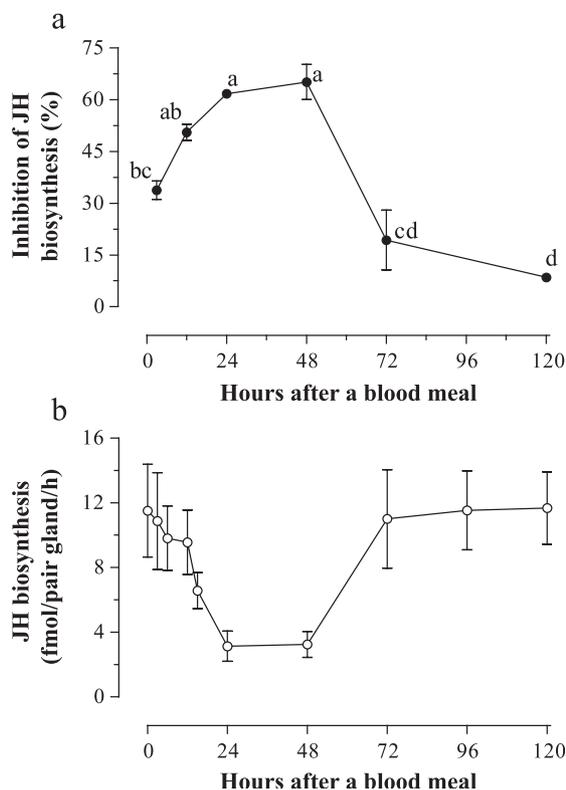
Fig. 4. The inhibitory activity of the brain extracts changes after blood feeding. (a) Effect of brain extracts: brains were dissected from females at different times after blood feeding. Extracts were tested on Br–CA–CC from 3-day-old sugar-fed females. Each Br–CA–CC was incubated with extracts from 10 brains. Each data point represents the mean ± S.E.M. of five independent determinations of individual CA complexes. Values labeled with the same letters are not significantly different at the 5% level by Tukey's test after ANOVA. (b) Biosynthesis of JH III in vitro after a blood meal: 3-day-old females were blood-fed. CA were dissected at different times after feeding and incubated for 4 h. Time-0 data point is for the 3-day-old sugar-fed females. Each data point represents the mean ± S.E.M. of 10–25 independent determinations of individual CA complexes (reproduced from Li et al. [10]).
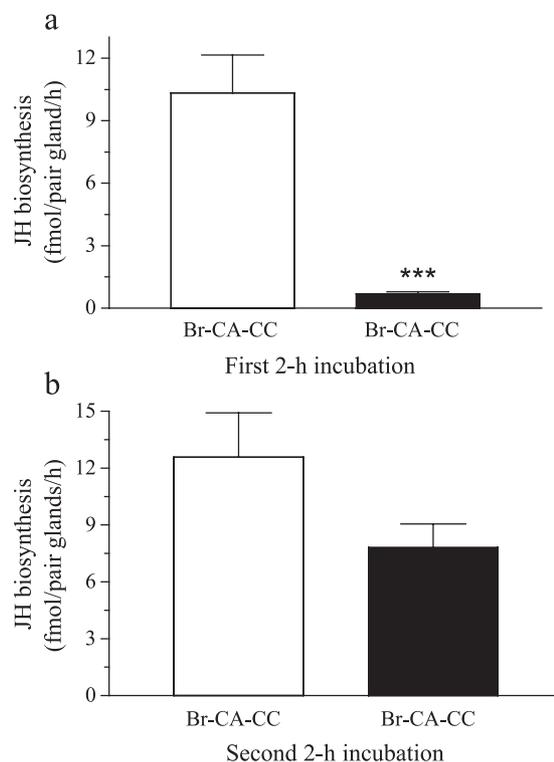
Fig. 5. Reversibility of the effect of inhibitory factor. Br–CA–CC from 3-day-old sugar-fed females were incubated for two successive 2-h periods. (a) In the first 2-h period, CA complexes were incubated in control medium containing labeled L-[methyl-^{3}H] methionine (open bars), or in medium containing labeled L-[methyl-^{3}H] methionine plus extracts from 10 brains dissected from 48 h blood-fed females (filled bars). After 2 h, the glands were rinsed in fresh culture medium M-199 without methionine for 30 min, and then (b) both groups (open and filled bars) were incubated for a second 2-h period in fresh medium M-199 containing labeled L-[methyl-^{3}H] methionine. Each data point represents the mean ± S.E.M. of five independent determinations of individual CA complexes. Asterisk indicates significant differences between control and treatments in respective group by unpaired t test at $P < 0.001$ (***).

difference in levels of JH release between experimental and control groups was found during the second incubation, indicating that the effect of the brain extracts is reversible (Fig. 5b). The same results were obtained when either isolated brains or preconditioned medium was used in place of brain extract (results not shown).

3.5. Initial characterization of the inhibitory fraction

Brain extracts were exposed to 100 °C for 10 min. The heated extracts maintained allatostatic activity and significantly inhibited JH biosynthesis (Table 1). However, the extracts lost their allatostatic activity after being exposed to proteinase K treatment for 4 h at 30 °C (Table 1).

3.6. Effect of YXFGL-amide- and PISCF-allatostatins

The basal rates of JH biosynthesis by Br–CA–CC dissected from 3-day-old sugar-fed females or females 24

Table 1
Effects of extracts of 10 brains dissected from females 48 h after blood feeding on JH biosynthesis by Br–CA–CC from 3-day-old sugar-fed females

Treatments	JH biosynthesis (fmol/pair gland/h)
Control	13.01 ± 2.06
Extract (no treatment)	4.06 ± 1.61**
Extract heated at 100 °C	5.78 ± 1.11*
Extract digested by proteinase K	14.42 ± 2.08

Three trials with five replicates were run for each treatment.

　* Indicates significant differences between control and respective group by unpaired t test at $P < 0.05$.

　** Indicates significant differences between control and respective group by unpaired t test at $P < 0.01$.

h after blood feeding were not significantly affected by addition to the incubation medium of any of the five individual *Aedes* YXFGL-amide-allatostatins (Aea-ASs) or a combination of the five Aea-ASs at 10^{-8} M each (Fig. 6a,b). The addition to the culture medium of 4×10^{-5} M FA resulted in significant increases in JH biosynthesis; however, the addition of any of the five Aea-ASs, individually or together, to these FA-stimulated CA did not significantly modify JH biosynthesis when compared to FA-stimulated controls (Fig. 6c,d). Synthesis of JH by isolated Br–CA–CC
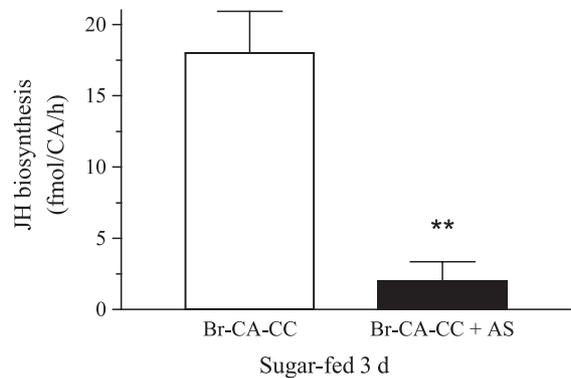

Fig. 7. Effect of *An. gambiae* PISCF-allatostatin on JH synthesis. Br–CA–CC dissected from 3-day-old sugar-fed female were incubated in medium (Br–CA–CC) or medium with 10^{-9} M *An. gambiae* PISCF-allatostatin (Br–CA–CC + AS). Each bar represents the mean $\pm$ S.E.M. of five independent determinations of individual CA complexes. Asterisk above the bar indicates the value is significantly higher than the Br–CA–CC by unpaired t test at $P < 0.01$(**).

from newly emerged females, from 1- and 5-day-old sugar-fed females and from females 1-, 12-, 48-, 72- and 96-h after blood feeding was not significantly affected by addition of individual or combined Aea-ASs at concentrations of 10^{-8} M (results not shown). In addition, Aea-ASs at concentra-
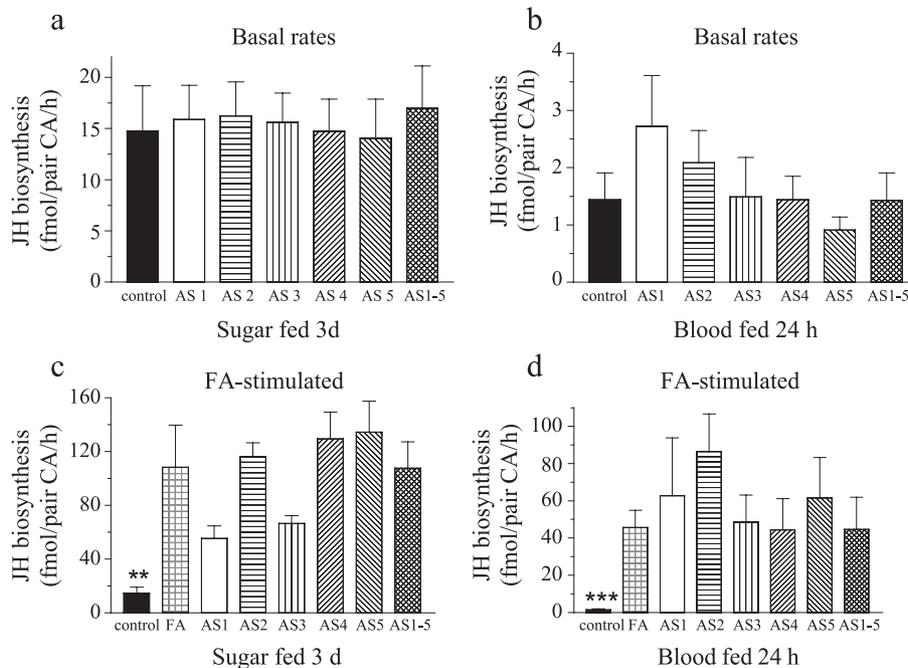

Fig. 6. Effect of *Aedes* YXFGL-amide-allatostatins on JH synthesis: (a) Br–CA–CC dissected from 3-day-old sugar-fed female were incubated in the medium without Aea-ASs (control) or with Aea-allatostatin 1 (AS1), Aea-allatostatin 2 (AS2), Aea-allatostatin 3 (AS3), Aea-allatostatin 4 (AS4), Aea-allatostatin 5 (AS5) or a combination of the five Aea-ASs (AS1–5) at a concentration of 10^{-8} M. (b) Br–CA–CC dissected from females 24 h after blood feeding and incubated in the medium without Aea-ASs (control) or with *Aedes* YXFGL-amide-allatostatins as described for (a). (c) Br–CA–CC dissected from 3-day-old sugar-fed female and incubated in medium with 4.0×10^{-5} M FA (FA) or 4.0×10^{-5} M FA plus *Aedes* YXFGL-amide-allatostatins at a concentration of 10^{-8} M as described for (a). (d) Br–CA–CC dissected from females 24 h after blood feeding and incubated in medium with 4.0×10^{-5} M FA or 4.0×10^{-5} M FA plus *Aedes* YXFGL-amide-allatostatins (as described for c). Each data point represents the mean $\pm$ S.E.M. of $10-25$ independent determinations of individual CA complexes. (a and b) No significant differences were found in comparison with control by ANOVA analysis ($P > 0.05$). (c and d) The addition of FA resulted in a significant increase of JH synthesis in sugar-fed ($P > 0.01$) and blood-fed ($P > 0.001$) in comparison with controls. No significant differences were found in FA stimulated + AS-treated in comparison with FA stimulated by ANOVA analysis ($P > 0.05$).

tions of 10^{-6} M had no significant effect on JH biosynthesis by Br−CA−CC dissected from 1-day sugar-fed females or from females 12 h after blood feeding (results not shown). Moreover, addition of Aea-ASs had no effect when tested on Br−CA−CC dissected from newly emerged female that were stimulated with *Aedes* allatotropin (Aea-AT) or Aea-AT + FA as described by Li et al. [7] or Br−CA−CC from 48-h-old sugar-fed or 36-h blood-fed females stimulated with Aea-AT (results not shown).

The addition of 10^{-9} M of synthetic *A. gambiae* PISCF-allatostatin significantly reduced JH biosynthesis by Br−CA−CC dissected from 3-day-old sugar-fed females (Fig. 7).

4. Discussion

These studies establish that there are factors present in the brain of mosquitoes that modulate CA activity. Isolation of the CA−CC from the brain caused a significant increase in JH synthesis by glands from females both before and after blood feeding. There was a significant reduction of JH synthesis when CA were incubated with isolated brains, in medium in which brains had been maintained (pre-conditioned medium), or with brain extracts, suggesting that allatostatin-like factors are present in the brain of the mosquito and released into the medium.

In the cockroach, *Diploptera punctata*, nervous inhibition of the CA by the brain is lost when the brain is removed, and CA connected to the brain produce less JH than isolated CA [18]. One class of factors responsible for inhibition of the CA is the cockroach YXFGL-amide-allatostatins [19]. Among other sources, allatostatins are produced in lateral cerebral neurosecretory cells that innervate the CA [2,19]. Presumably, allatostatins are released from the axon endings of these cells and exert their effect directly on the CA [20]. Allatostatins have been identified in cockroach hemocytes and have been shown to act on the CA via the hemolymph as well [21].

This study demonstrates that allatostatic factor(s) exist in extracts of brains from blood-fed females, whose CA synthesize/release JH at a low rate when incubated as a complex with the brain. The allatostatic factor(s) is heat stable at 100 °C for 10 min and is digested by proteinase K, suggesting a peptidic nature.

The inhibition of CA activity observed with either extract or pre-conditioned medium was reversible. This type of reversible effect was described by Unnithan et al. [22] as a "dynamic response", that is, one that can be measured readily in vitro, is normally reversible because the activity of the effecter can be washed out of the in vitro system, and the effecter must be continually renewed to maintain the effect. This "dynamic response" is characteristic of most of the identified allatostatins and appears to be characteristic of the factor from the *Aedes* brain.

To identify allatostatic factors in *Ae. aegypti*, the effects of members of the two families of allatostatins described in mosquitoes thus far were assayed using *Ae. aegypti* Br−

CA−CC complexes: *An. gambiae* PISCF-allatostatin (homolog to Manduca PISCF-allatostatin) and YXFGL-amide-allatostatins (homologs to cockroach YXFGL-amide-allatostatins). The addition of physiological concentrations of synthetic *A. gambiae* PISCF-allatostatin (10^{-9} M) to the medium significantly reduced JH biosynthesis by the CA in vitro. This is the first 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 other holometabolous insects.

In addition, in this study, we systematically investigated the effects of five native *Aedes* YXFGL-amide-allatostatins on JH synthesis by the CA during a reproductive cycle, either alone and in combination, at both physiological and pharmacological concentrations. The effect of Aea-AS on CA stimulated by addition of FA and/or *Ae. aegypti* allatotropin was also examined. The results of all these experimental permutations, although negative, conclusively demonstrate for the first time that the cockroach YXFGL-amide-allatostatins are not involved in the regulation of JH synthesis in mosquitoes at this stage in the life cycle.

In summary, factors present in the brain of the adult mosquito modulate the activity of the CA. Furthermore, a PISCF-allatostatin, but not the YXFGL-amide class of allatostatins, might regulate JH synthesis in mosquito. Additional, unidentified allatostatins-like factors may exist and be responsible for regulating rates of JH biosynthesis during the gonotrophic cycle, especially after blood-feeding [9,10,23]. A combination of chromatographic and immunological techniques will be used to further characterize the inhibitory factor(s) present in mosquito heads and the role of PISCF-allatostatin.

References

[1] Gilbert LI, Granger NA, Roe RM. The juvenile hormones: historical facts and speculations on future research directions. Insect Biochem Mol Biol 2000;30:617–44.
[2] Stay B. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. Insect Biochem Mol Biol 2000;30:653–62.
[3] Stay B, Tobe SS, Bendena WG. Allatostatins-identification, primary structures, functions and distribution. Adv Insect Physiol 1994;25: 267–337.
[4] Tobe SS, Garside CS, Jansons IS, Price MD, Bendena WG. In: Suzuki A, Kataoka H, editors. Molecular Mechanisms of Insect Metamorphosis and Diapause. Tokyo: Matsumoto S., Ind. Publishing and Consulting, 1995. p. 13–24.

[5] Duve H, Thorpe A, Johnsen AH, Maestro JL, Scott AG, East PD. In: Coast GM, Webster SG, editors. Recent Advances in Arthropod Endocrinology, vol 65. Society for Experimental Biology; 1998. p. 229–47. Seminar series.

[6] Weaver RJ, Edwards JP, Bendena WG, Tobe SS. In: Coast GM, Webster SG, editors. Recent Advances in Arthropod Endocrinology, vol 65. Society for Experimental Biology; 1998. p. 3–32. Seminar series.

[7] Li Y, Unnithan GC, Veenstra JA, Feyereisen R, Noriega FG. Stimulation of JH biosynthesis by the corpora allata of adult female *Aedes aegypti* in vitro: effect of farnesoic acid and *Aedes* allatotropin. J Exp Biol 2003;206:1825–32.

[8] Bendena WG, Donly BC, Tobe SS. Allatostatins: a growing family of neuropeptides with structural and functional diversity. Ann NY Acad Sci 1999;897:311–29.

[9] Shapiro AB, Wheelock GD, Hagedorn HH, Baker FC, Tsai LW, Schooley DA. Juvenile hormone and juvenile hormone esterase in adult females of the mosquito *Aedes aegypti*. J Insect Physiol 1986;32:867–77.

[10] Li Y, Hernandez-Martinez S, Unnithan GC, Feyereisen R, Noriega FG. Activity of the corpora allata of adult female *Aedes aegypti*: effects of mating and feeding. Insect Biochem Mol Biol 2003; 33:1307–15.

[11] Veenstra JA, Noriega FG, Graf R, Feyereisen R. Identification of three allatostatins and their cDNA from the mosquito *Aedes aegypti*. Peptides 1997;18:937–42.

[12] Riehle MA, Garczynski SF, Crim JW, Hill CA, Brown MR. Neuropeptides and peptide hormones in *Anopheles gambiae*. Science 2002;298:172–5.

[13] Noriega FG, Colonna AE, Wells MA. Increase in the size of the amino acid pool is sufficient to activate translation of early trypsin mRNA in *Aedes aegypti* midgut. Insect Biochem Mol Biol 1999;29: 243–7.

[14] Feyereisen R, Tobe SS. A rapid partition assay for routine analysis of JH release by insect CA. Anal Biochem 1981;111:372–5.

[15] Feyereisen R. Radiochemical assay for juvenile hormone III biosynthesis in vitro. In: Law JH, Rilling HC, editors. Methods Enzymology, vol. III. Orlando, FL: Academic Press; 1985. p. 530–9.

[16] Hammock BD, Abdel-Aael YAI, Mullin CA, Hanzlik TN, Roe RM. Substituted thiotrifluoropropanones as potent selective inhibitors of juvenile hormone esterase. Pestic Biochem Physiol 1984;22:209–23.

[17] Veenstra JA, Costes L. Isolation and identification of a peptide and its cDNA from the mosquito *Aedes aegypti* related to *Manduca sexta* allatotropin. Peptides 1999;20:1145–51.

[18] Rankin SM, Stay B, Aucoin RR, Tobe SS. In vitro inhibition of juvenile hormone synthesis by corpora allata of the viviparous cockroach, *Diploptera punctata*. J Insect Physiol 1986;32:151–6.

[19] Stay B, Fairbairn S, Yu CG. Role of allatostatins in the regulation of juvenile hormone synthesis. Arch Insect Biochem Physiol 1996;32: 287–97.

[20] Lloyd GT, Woodhead AP, Stay B. Release of neurosecretory granules within the corpus allatum in relation to the regulation of juvenile hormone synthesis in *Diploptera punctata*. Insect Biochem Mol Biol 2000;30:739–46.

[21] Skinner JR, Fairbairn SE, Woodhead AP, Bendena WG, Stay B. Allatostatin in hemocytes of the cockroach *Diploptera punctata*. Cell Tissue Res 1997;290:119–28.

[22] Unnithan GC, Sutherland TD, Cromey DW, Feyereisen R. A factor causing stable stimulation of juvenile hormone synthesis by *Diploptera punctata* corpora allata in vitro. J Insect Physiol 1998;44: 1027–37.

[23] Borovsky D, Carlson DA, Ujvary I. In vivo and in vitro biosynthesis and metabolism of methyl farnesoate, juvenile hormone III, and juvenile hormone III acid in the mosquito *Aedes aegypti*. J Med Entomol 1992;29:619–29.