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Stimulation of JH biosynthesis by the corpora allata of adult female *Aedes aegypti in vitro*: effect of farnesoic acid and *Aedes* allatotropin

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Summary

Previous studies have demonstrated that the synthesis of juvenile hormone (JH) by the isolated corpora allata (CA) complex *in vitro* as well as the JH titer in the yellow fever mosquito *Aedes aegypti* are elevated before feeding and low after a blood meal. In the present study, we used an *in vitro* radiochemical assay to analyze the effect of farnesoic acid (FA) and *Aedes* allatotropin (Aedes-AT) on the biosynthesis of JH and methyl farnesoate (MF) by the isolated CA complex of *A. aegypti* adult female. CA complex from day-0 females (0–1 h after emergence) exhibited a low basal juvenile hormone III (JH III) biosynthetic activity and did not respond to either allatotropic or FA stimulation. However, incubation of CA complexes from newly emerged females with Aedes-AT plus FA resulted in very high production of JH III. This is the first report suggesting that allatotropin makes corpora allata in newly emerged females capable for JH biosynthesis. When we studied CA complexes dissected from females 1 day after emergence, the stimulatory action of Aedes-AT was strong and dose-dependent, with maximum stimulation in the range of 10^{-8}–10^{-9} mol l^{-1}, suggesting that Aedes-AT is indeed a true allatotropin (a molecule with allatotropic activity) in *A. aegypti.* The addition to the culture medium of 40 μmol l^{-1} FA, a JH precursor, resulted in a 9-fold increase in JH III biosynthesis in 2-, 4- and 6-day-old sugar-fed females. The two major labeled products synthesized by the stimulated CA complex were identified as JH III and MF by RP-HPLC and GC–MS. Treatment of CA complexes with FA, but not Aedes-AT, resulted in an increase in MF. Application of both Aedes-AT and FA to the CA complexes of 2-, 4- and 6-day-old females resulted in the same effects as FA alone. These data suggest that in sugar-fed females, FA and Aedes-AT exert different effects on the terminal steps in JH biosynthesis.

Key words: mosquito, *Aedes*, farnesoic acid, allatotropin, corpora allata, regulation, juvenile hormone.

Introduction

Juvenile hormones (JHs) are a class of regulatory sesquiterpenoids that control metamorphosis in immature insects and reproduction in most 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 (Gilbert et al., 2000; Stay, 2000).

JH levels in adult female *A. aegypti* were measured by Shapiro et al. (1986). JH levels increase during the first two days after adult emergence and remain high before feeding. When a female takes a blood meal, the JH level falls rapidly during the first three hours and reaches its lowest point 24 h after the blood meal. Forty-eight hours after the blood meal, the JH level starts to rise, and after 96 h it is equivalent to the pre-blood meal value. JH titer is essentially determined by the rate at which the CA synthesizes JH. The *in vitro* and *in vivo* biosynthesis of JH in *A. aegypti* was studied by Borovsky and collaborators (Borovsky and Carlson, 1992; Borovsky et al., 1992, 1994a,b); they developed an 'exposed corpus allata' assay, i.e. the head–thorax complex was incubated in the presence of different radioactive precursors [methyl farnesoate (MF) and methionine].

Two groups of peptides, allatotropins (AT) and allatostatins (AS), which either stimulate or inhibit JH synthesis, respectively, have been found in insects from different orders. Although allatotropic activity has been described in biological extracts from a number of species, such as the larvae of the wax moth *Galleria mellonella* (Bogus and Scheller, 1996), adult locust *Locusta migratoria* (Gadot et al., 1987; Lehmberg et al.,

1992), crickets *Gryllus bimaculatus* and *Acheta domesticus* (Lorenz and Hoffmann, 1995), true bug *Pyrrhocoris apterus* (Hodkova et al., 1996), Loreyi leafworm *Mythimna loreyi* (Kou and Chen, 2000) and the honey bee *Apis mellifera* (Gäde et al., 1997), so far only a single peptide has been chemically identified. This is the *Manduca sexta* allatotropin (Mas-AT) identified from heads of the pharate adult tobacco hornworm *Manduca sexta* (Kataoka et al., 1989), a 13-residue amidated peptide with the sequence GFKNVEMMTARGFamide that stimulates adult CA *in vitro* but does not affect the activity of larval or pupal CA (Kataoka et al., 1989). Mas-AT was also purified from the methanolic brain extracts of the fall armyworm *Spodoptera frugiperda* (Oeh et al., 2000). It also stimulates JH biosynthesis in other lepidopteran species, such as the tobacco budworm *Heliothis virescens* (Kataoka et al., 1989), the tomato moth *Lacanobia oleracea* (Audsley et al., 1999, 2000) and *S. frugiperda* (Oeh et al., 2000), and non-lepidopteran species, such as *A. mellifera* larvae (Rachinsky and Feldlaufer, 2000; Rachinsky et al., 2000) and the black blowfly *Phormia regina* (Tu et al., 2001). By use of immunochemical and molecular techniques, Mas-AT has been shown to be present in the brain of larval *L. oleracea* and the cotton leafworm *Spodoptera littoralis* (Audsley et al., 1999, 2000), the abdominal nervous system of the cockroaches *Leucophaea maderae* and *Periplaneta americana* (Rudwall et al., 2000), the true armyworm moth *Pseudaletia unipuncta* (Truesdell et al., 2000), *P. regina*, the adult large milk-weed bug *Oncopeltus fasciatus*, the adult oriental fruit fly *Dacus dorsalis*, larval and adult *M. loreyi* and the larval tea silk moth *Andraca bipunctata* (Tu et al., 2002). In addition, cDNAs encoding Mas-AT have been cloned from *M. sexta* (Taylor et al., 1996), *P. unipuncta* (Truesdell et al., 2000) and the silk moth *Bombyx mori* (Park et al., 2002).

Besides stimulating JH biosynthesis, Mas-AT displays a multifunctional character in *M. sexta* and other insects, including inhibition of ion transport in *M. sexta* midgut (Lee et al., 1998), stimulation of foregut contractions in the moths *Helicoverpa armigera* (at extremely low concentrations) and *L. oleracea* (Duve et al., 1999, 2000) and acceleration of heart rate in *L. maderae*, *P. americana* (Rudwall et al., 2000), pharate adult *M. sexta* (Veenstra et al., 1994) and *P. unipuncta* (Koladich et al., 2002). Mas-AT also plays a role in circuits relaying photic information from circadian photoreceptors to the central pacemaker in *L. maderae* (Petri et al., 2002).

Recently, immunocytochemistry using an antiserum to MAS-AT revealed that an allatotropin-immunoreactive peptide is present in the abdominal ganglia of the mosquito *Aedes aegypti* (Veenstra and Costes, 1999). The allatotropin-immunoreactive peptide was isolated and its structure determined to be APFRNSEMMTARGFamide (Aedes-AT); in addition, a cDNA encoding this peptide was cloned (Veenstra and Costes, 1999).

In the present study, we demonstrate the stage-specific stimulatory effects of Aedes-AT and of the JH precursor farnesoic acid (FA) on adult female *A. aegypti* CA activity *in vitro*.

Materials and methods

Chemicals

Synthetic Aedes-AT was custom-synthesized at the Center for Biotechnology Research, Kansas State University. The synthesized Aedes-AT was purified by reversed-phase liquid chromatography and assessed to be ≥97% pure by analytical reversed-phase liquid chromatography, mass spectroscopy and amino acid analysis. (*E*,*E*)-methyl farnesoate (MF) and (*E*,*E*)-farnesoic acid (FA) were purchased from Echelon (Salt Lake City, UT, USA), and JH III was purchased from Sigma (St Louis, MO, USA) or ICN (Irvine, CA, USA). HPLC-grade ethyl acetate, hexane and methanol were from Burdick and Jackson (Muskegon, MI, USA).

Insects

Aedes aegypti (L.) of the Rockefeller strain were reared at 28°C and 80% relative humidity under a photoperiod cycle of 16 h:8 h light:dark as previously described (Noriega et al., 1999). The newly emerged females were collected within 1 h of emergence, and during that period females did not feed. For females older than 1 h (i.e. sugar-fed females), a 3.0% sucrose solution was provided *ad libitum*. Only virgin females were used in this study.

In vitro *radiochemical assay for CA activity*

For preparation of isolated CA complexes, mosquitoes were immobilized by brief exposure to ice. After cutting off the legs, wings, antennae and abdomen, the anterior half of the body was pinned to a silicon dissecting dish and covered with a drop of mosquito saline buffer (138 mmol l^{-1} NaCl, 8.4 mmol l^{-1} KCl, 4 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $MgCl_2$, 12 mmol l^{-1} NaH_2PO_4, 12 mmol l^{-1} Na_2HPO_4 and 42.5 mmol l^{-1} sucrose). The thorax was split open and the corpora allata (CA) plus corpora cardiaca (CC) complex attached to the aorta was 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. This facilitated the visualization and transfer of the complexes. In all the experiments described in this paper we used CA + CC + aorta + brain + head capsule preparations, which we will refer to as 'CA complexes'.

After dissection, the CA complexes were held in tissue culture medium M-199 (Specialty Media, Phillipsburg, NJ, USA) without methionine, containing 2% Ficoll 400 and 25 mmol l^{-1} Hepes (pH 6.5). After a pre-incubation of 1–2 h to consume intraglandular methionine, the CA complexes were transferred into a carbowax-coated flat-bottomed glass tube containing 100 μl of sterile tissue culture medium M-199 with 25 mmol l^{-1} Hepes (pH 6.5) and 2% Ficoll 400 containing L-[methyl-^{3}H]methionine [specific activity 2.96–3.11 TBq $mmol^{-1}$ (80–84 Ci $mmol^{-1}$); Amersham Pharmacia, Piscataway, IL, USA] as described by Feyereisen and Tobe (1981) and Feyereisen (1985). The final concentration of methionine in the medium was 50 μmol l^{-1}, and the specific activity was 0.56 TBq mmol l^{-1} (15 Ci mmol l^{-1}). Under these conditions,

the incorporation of L-[methyl-^{3}H]methionine into JH III was linear for at least 6 h.

CA were cultured in the dark at 30°C for 4 h under continuous gentle agitation on an ADAMS™ Nutator Mixer (Becton-Dickinson, Franklin Lakes, NJ, USA). Incubations were terminated by the addition of 100 µl 1% EDTA, and 100 µl methanol containing 25 µg each cold JH III and MF were used as carriers and internal standards. At the end of the experiment, the incubation medium and the gland were extracted together with methanol/hexane (1:10 v/v) and were separated by thin-layer chromatography (TLC). After TLC separation [developed in 2:1 (v/v) hexane/ethyl acetate], the JH and MF bands were detected under UV light, cut, put into 10 ml scintillation cocktail overnight and assayed for ^{3}H. The quantities of JH and MF produced in the experiment were then calculated from the specific activity of the L-[methyl-^{3}H]methionine in the medium, assuming a specific incorporation ratio of 1 (non-isotopic dilution).

HPLC and mass spectral analysis of products synthesized by CA complexes

Detection of radiolabeled products was done using a Beckman Gold HPLC system model 126, with an Ultrasphere® C18 reverse-phase HPLC column (250×4.6 mm, 5 µm particles) and a scanning model 167 UV detector set at 214 nm. The column was eluted using a linear gradient of 40–100% acetonitrile in H_2O. The separating conditions were as follows: solvent flow rate, 1 ml min^{-1}; gradient, 0–5 min 40% CH_3CN, 5–45 min 40–100% CH_3CN (linear gradient), 45–50 min 100% CH_3CN, 50–55 min, 40% CH_3CN. Data from HPLC were detected and analyzed using the Beckman Gold system software. The recovery calculated from the UV traces was usually 70–98%. 1 ml fractions were diluted with 10 ml scintillation cocktail and analyzed for ^{3}H.

The peaks from HPLC were analyzed by chemical ionization mass spectroscopy (MS) using a Finnigan-Matt ITS 40® ion trap MS interfaced to a Varian Star 3400® gas chromatograph with a cool-on-column injector as described by Teal et al. (2000). Identification of JH homologs was based on comparison of fragmentation patterns (60–300 amu) and retention indexes of compounds eluting during analysis of natural product samples with those of synthetic standards.

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 (GraphPad Software; San Diego, CA, USA). The results were expressed as means ± S.E.M. and considered significantly different at $P<0.05$. Values of percentage stimulation of JH synthesis by treatment were calculated *via* the following formula: 100 × (activity with allatotropin or FA – activity without allatotropin or FA) / activity without allatotropin or FA.

Results

Synthesis of JH by Aedes aegypti *CA complexes* in vitro *in the presence of exogenous farnesoic acid and allatotropin*

Corpora allata from 4-day-old sugar-fed females were dissected and incubated in M-199 media containing L-[methyl-^{3}H]methionine. The addition to the culture medium of 40 µmol l^{-1} FA or 10^{-9} mol l^{-1} Aedes-AT stimulated increases in JH and MF biosynthesis in the CA complexes. *In vitro*-biosynthesized compounds were identified by HPLC. We found only two major radioactive peaks; elution of the two radioactive fractions coincided exactly with the peaks for the MF and JH III internal standards (retention times: 22.46 min for JH III and 36.60 min for MF). Standards for JH I and JH II were also used, and no evidence for the presence of other JH homologs was found in any of the samples. Biosynthesis of JH III bisepoxide (JHB_3) by the CA and accessory gland of male *A. aegypti* has been reported (Borovsky et al., 1994a). Under the HPLC conditions used in our studies, JHB_3 should have eluted before JH III due to the decreased lipophilicity caused by the addition of a second epoxide group (Richard et al., 1989; Borovsky et al., 1992). After HPLC purification, the JH III fraction was analyzed by GC–MS. *In vitro*-biosynthesized JH III identity was confirmed based on the diagnostic ions of the standard JH III; *m*/*e*=111, 147, 189, 217 and 235.

Dose–response relationship between exogenous FA and JH and MF biosynthesis in vitro

The CA complexes from insects with high basal JH III biosynthetic activity (2-day-old sugar-fed females) were incubated in M-199 media containing L-[methyl-^{3}H]-methionine and various concentrations of FA. Fig. 1A shows that, in the absence of FA, the rate of JH III biosynthesis was 15.56 fmol complex^{-1} h^{-1}. JH III biosynthesis was markedly stimulated (approximately 9-fold) in the presence of 10 µmol l^{-1} FA, and this stimulation remained constant when the concentration of FA was increased up to 160 µmol l^{-1}. On the other hand, when we used CA complexes from insects with low basal JH III biosynthetic activity (4-day-old sugar-fed females), JH III biosynthesis augmented with FA increases up to a concentration of 40 µmol l^{-1} FA and then remained constant when the concentration of FA was further increased up to 160 µmol l^{-1} (Fig. 1B).

CA complexes of 2-day-old sugar-fed females accumulated MF in the presence of FA. There was a linear relationship between MF increases and FA concentration in the medium (r^2=0.86). A similar trend was found in 4-day-old sugar-fed females (r^2=0.92). Maximal stimulation occurred at an FA concentration of 40 µmol l^{-1}, and so in all subsequent experiments a concentration of 40 µmol l^{-1} FA was used.

Stimulation of JH biosynthesis by Aedes-AT

The effect of increasing concentrations of Aedes-AT (10^{-12}–10^{-6} mol l^{-1}) was tested on glands from 1-day-old (high basal activity; Fig. 2A) and 3-day-old (low basal activity; Fig. 2B) sugar-fed females. Fig. 2C shows that high rates of JH

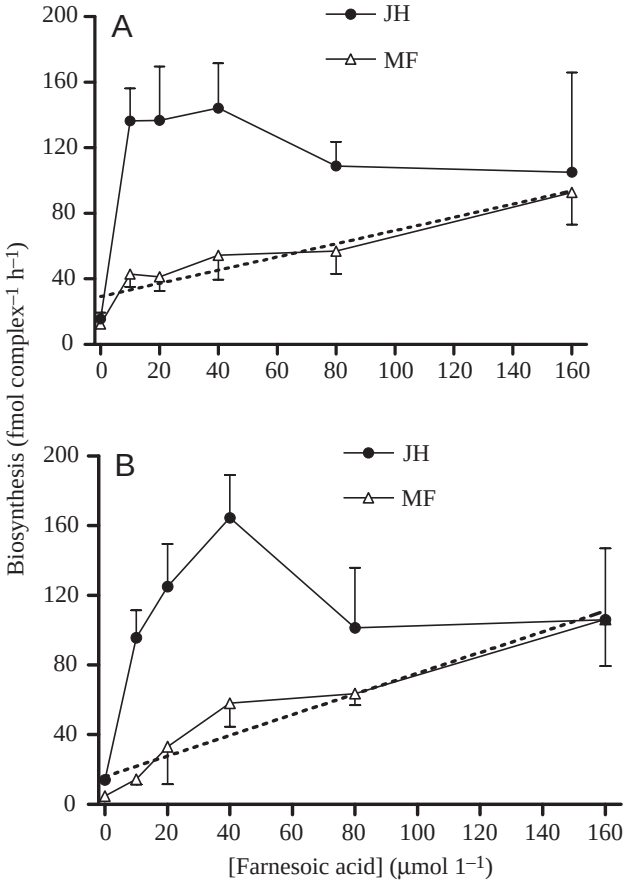

Fig. 1. Effect of farnesoic acid (FA) on biosynthesis of juvenile hormone (JH) and methyl farnesoate (MF). (A) Corpora allata (CA) complexes from 2-day-old sugar-fed females. (B) CA complexes from 4-day-old sugar-fed females. CA complexes were incubated *in vitro* with 10–160 μmol l^{-1} FA. Each data point represents the mean ± S.E.M. of 5–25 independent determinations of individual CA complexes. The broken line represents the linear relationship between the FA added and the MF accumulation rate.

III biosynthesis (>50% increase) were observed over a narrow range of Aedes-AT concentrations (10^{-9}–10^{-8} mol l^{-1}) in 1-day-old sugar-fed females, and over a wider range (10^{-10}–10^{-6} mol l^{-1}) in 3-day-old sugar-fed females. MF biosynthesis was similar in CA treated with Aedes-AT and in controls when glands from 1-day-old and 3-day-old females were used (Fig. 2A,B). Maximal stimulation occurred at an Aedes-AT concentration of 10^{-9} mol l^{-1}, and so in all subsequent experiments a concentration of 10^{-9} mol l^{-1} Aedes-AT was used.

Response of CA to Aedes-AT in vitro

Aedes-AT did not affect JH production in CA dissected from newly emerged females and females 12 h after emergence. However, incubation of CA complexes from 1-day-old sugar-fed females with Aedes-AT resulted in a significant increase in rates of JH biosynthesis (Fig. 3A). Aedes-AT had no effect on JH production on CA from 2-day-

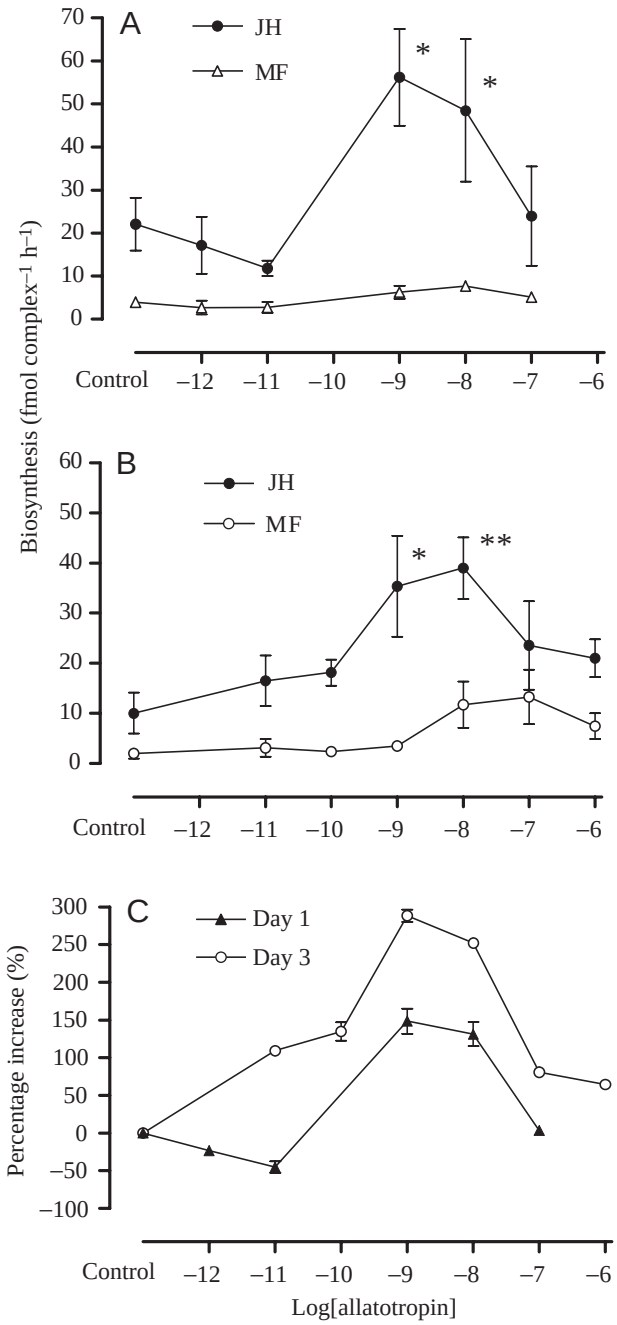

Fig. 2. Dose–response effect of *Aedes aegypti* allatotropin (Aedes-AT) on biosynthesis of juvenile hormone (JH) and methyl farnesoate (MF). (A) Corpora allata (CA) complexes from 1-day-old sugar-fed females. (B) CA complexes from 3-day-old sugar-fed females. CA complexes were incubated *in vitro* with 10^{-12}–10^{-6} mol l^{-1} Aedes-AT. Each data point represents the mean ± S.E.M. of 5–15 independent determinations of individual CA complexes. Asterisks denote significant differences from control values (unpaired *t*-test; **P*≤0.05; ***P*≤0.01). (C) Comparative analysis of the effect of Aedes-AT on 1- and 3-day-old sugar-fed females, expressed as percentage of JH increase over the controls. Each data point represents the mean ± S.E.M. of three independent calculations. Some of the S.E.M. are too small to show on this scale.

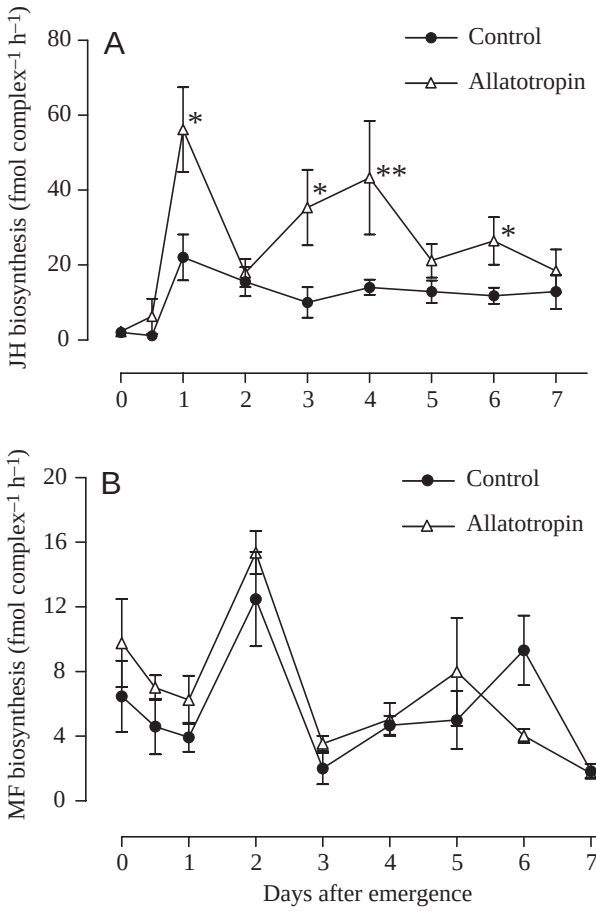

Fig. 3. Age-specific effect of *Aedes aegypti* allatotropin (Aedes-AT) on biosynthesis of (A) juvenile hormone (JH) and (B) methyl farnesoate (MF). Corpora allata (CA) complexes were incubated *in vitro* with 10^{-9} mol l^{-1} Aedes-AT. Each data point represents the mean ± S.E.M. of 5–25 independent determinations of individual CA complexes. Asterisks denote significant differences from respective control values (unpaired *t*-test; **P*≤0.05; ***P*≤0.01).

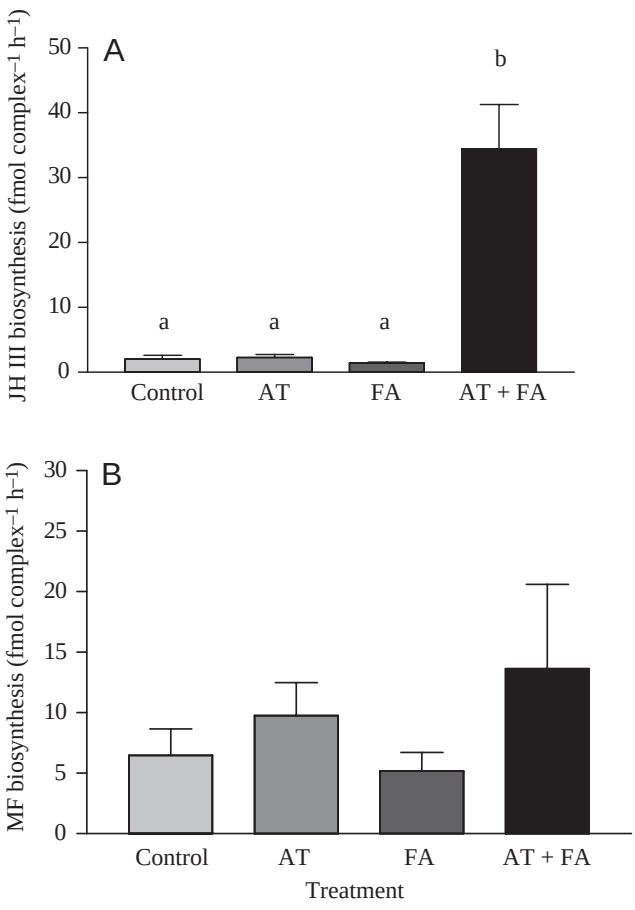

Fig. 4. Effects of *Aedes aegypti* allatotropin (Aedes-AT) and farnesoic acid (FA) on corpora allata (CA) activity of newly emerged females (0–1 h). CA complexes were incubated *in vitro* with 10^{-9} mol l^{-1} Aedes-AT or 40 μmol l^{-1} FA or a combination of both. (A) Juvenile hormone (JH) levels. (B) Methyl farnesoate (MF) levels. Each data point represents the mean ± S.E.M. of 5–25 independent determinations of individual CA complex. Values labeled with different letters are significantly different by Tukey's test after analysis of variance (ANOVA) at $P<0.05$.

old sugar-fed females, but CA complexes from 3-, 4- and 6-day-old sugar-fed females showed significantly higher JH levels in response to Aedes-AT; the peptide did not affect JH biosynthesis on CA from 5- and 7-day-old sugar-fed females. Aedes-AT did not induce significant increases in MF biosynthesis by CA complexes when compared with controls (Fig. 3B)

Allatotropin makes CA capable for JH production

CA complexes were dissected from females within 1 h of emergence and were incubated with FA (40 μmol l^{-1}), Aedes-AT (10^{-9} mol l^{-1}) or both. CA complexes from newly emerged females showed a very low basal level of JH biosynthesis *in vitro*. Biosynthesis of JH III in the presence of either FA or Aedes-AT was also very low and similar to those from unstimulated CA complexes (approximately 2.03 fmol complex^{-1} h^{-1}). However, incubation of CA complexes from newly emerged females with Aedes-AT plus FA resulted in a 17-fold increase in the production of JH III (35 fmol complex^{-1} h^{-1}; Fig. 4A). FA, Aedes-AT or both did not result in significant increases of MF levels when compared with controls (Fig. 4B).

Effect of Aedes-AT in the presence of FA

Simultaneous application of Aedes-AT and FA to the CA complexes of 0.5-, 2-, 4- and 6-day-old females had the same effects as applying FA alone (Fig. 5). Addition of Aedes-AT plus FA resulted in a large increase in JH biosynthesis in all cases (Fig. 5A). MF increases in the CA complex treated with FA or FA plus Aedes-AT were elevated when compared with untreated controls (Fig. 5B); however, MF from the CA complexes treated with FA was higher than from CA that had been treated with FA plus Aedes-AT (Fig. 5B).

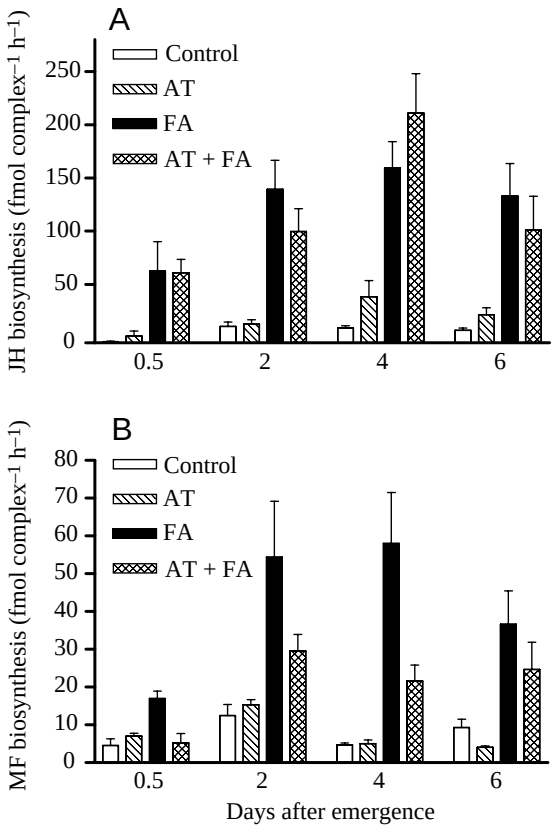

Fig. 5. Age-specific effect of *Aedes aegypti* allatotropin (Aedes-AT), farnesoic acid (FA) and Aedes-AT plus FA on corpora allata (CA) activity of sugar-fed females. CA complexes were incubated *in vitro* with 10^{-9} mol l^{-1} Aedes-AT or 40 μmol l^{-1} FA or a combination of both (AT + FA). (A) Juvenile hormone (JH) levels. (B) Methyl farnesoate (MF) levels. Bars represent the means ± S.E.M. of 5–15 independent replicates of individual CA complexes.

Discussion

JH III is synthesized by Aedes-AT and FA-stimulated CA complexes

Aedes-AT and FA stimulated significant increases in JH biosynthesis by the *A. aegypti* CA complexes *in vitro*. Using HPLC and GC–MS, we confirmed previous observations with unstimulated glands that showed that JH III is the only significant JH homolog found in *A. aegypti* females (Borovsky et al., 1992). We did not detect any JH bisepoxide as described in other diptera (Lefevere et al., 1993). Incubation of *Heliothis virescens* CA with Mas-AT stimulated significant increases in production of all three homologs, although changes of JH I and JH II levels were larger than JH III (Teal, 2002).

Responsiveness of CA complexes to FA

Exogenous FA, at optimal concentrations in the range of 20–40 μmol l^{-1}, is utilized by corpora allata *in vitro* to enhance rates of JH synthesis (Feyereisen et al., 1984; Tobe and Stay, 1985; Gadot and Applebaum, 1986). The addition of FA to the incubation medium containing isolated corpora allata results in a significant stimulation of JH biosynthesis in *Schistocerca gregaria*, *P. americana*, *D. punctata* (Pratt et al., 1975; Feyereisen et al., 1981), *Tenebrio molitor* (Weaver et al., 1980), *M. sexta* (Unni et al., 1991) and *A. mellifera* (Rachinsky et al., 2000). Moreover, Borovsky et al. (1994b) found that mosquito *A. aegypti* ovary can synthesize JH III from FA *in vivo* and *in vitro*.

The terminal two steps in JH III biosynthesis are: (1) the methylation of FA to MF, catalyzed by *O*-methyl transferase, and (2) an epoxidation of MF to JH III, catalyzed by methyl farnesoate epoxidase (Tobe and Stay, 1985). The addition of 40 μmol l^{-1} FA to CA dissected from newly emerged female *A. aegypti* showed no stimulation in MF or JH III production (Fig. 4). However, CA dissected from sugar-fed females 12 h after emergence showed an enormous (60-fold) increase in JH III biosynthesis. A 9-fold increase in JH III biosynthesis was found in CA dissected from sugar-fed females 2, 4 and 6 days after emergence. These results suggest that at least two terminal enzymes for JH biosynthesis are not activated in newly emerged females, *O*-methyl transferase and methyl farnesoate epoxidase; they are progressively activated when the CA mature, and after this maturation is completed they are kept at a relatively high level. The same phenomenon was described in newly emerged (0–1 day) adult female *L. migratoria* (Gadot and Applebaum, 1986).

In CA from 2- and 4-day-old sugar-fed female *A. aegypti*, an increase in FA concentration (more than 40 μmol l^{-1}) resulted in a linear increase of MF in the glands without causing a significant increase in JH biosynthesis. This observation suggests that such high FA concentrations saturate the methyl farnesoate epoxidase, a situation that probably does not occur under physiological conditions.

MF accumulation in the corpora allata also occurred in other insects under certain experimental conditions. Specific inhibition of methyl farnesoate epoxidase activity by 1,5-disubstituted imidazoles resulted in an increase in MF content in *D. punctata* (Unnithan et al., 1995). Elevated concentrations of exogenous FA led to an accumulation of MF in corpora allata from *P. americana* (Pratt et al., 1975). In *G. bimaculatus*, MF accumulation was observed after treatment of the glands with either 20 μmol l^{-1} or 200 μmol l^{-1} farnesol (Wennauer and Hoffmann, 1988). In most insects, however, FA stimulation does not result in MF accumulation, suggesting that the epoxidative capacity of the corpora allata is generally greater than their capacity for the esterification. Meanwhile, under unstimulated conditions, there is no such increase of MF in sugar-fed *A. aegypti* females. Thus, two different mechanisms seem to be responsible for the low rate of JH production by the sugar-fed *A. aegypti* 2 days after emergence: (1) a limitation in the production of JH precursors and (2) a rate limitation in the terminal step of JH biosynthesis.

Responsiveness of corpora allata complex to Aedes-AT

In the present study, Aedes-AT stimulated maximal JH biosynthesis *in vitro* (up to 6-fold) in 0.5- and 3-day-old sugar-fed virgin females of *A. aegypti* (Figs 3, 4). These maximal

levels of stimulation for Aedes-AT were similar to those previously found for Mas-AT during *in vitro* experiments using CA of various insects: up to 2.2-fold increase of JH in *A. mellifera* (Rachinsky et al., 2000), up to three times increase in larval *L. oleracea* (Audsley et al., 2000), a 2.6-fold increase in sugar-fed adult females of *P. regina* (Tu et al., 2001) and up to a 6-fold increase in adult *M. sexta* (Kataoka et al., 1989). However, the maximal level of stimulation by Aedes-AT in *A. aegypti* was lower than those by Mas-AT in either *S. frugiperda* (approximately 7-fold; Oeh et al., 2000) or in day 0 (approximately 15-fold) and day 6 (approximately 10-fold) adult virgin female of *P. unipuncta* (Koladich et al., 2002).

In adult sugar-fed virgin *A. aegypti*, Aedes-AT exhibited a stage-specific stimulation of JH biosynthesis in a dose-dependent manner, with a maximal concentration of approximately 10^{-8}–10^{-9} mol l^{-1}; a concentration similar to the titer of the peptide found in the hemolymph of most insect species [10^{-6} mol l^{-1} in *P. unipuncta* and 10^{-8} mol l^{-1} in *L. oleracea* and *P. regina* (Audsley et al., 2000; Koladich et al., 2002; Tu et al., 2001)]. Taken together, these results suggest that Aedes-AT is indeed a true allatotropic factor in *A. aegypti*.

Aedes-AT stimulation of JH and MF synthesis in the presence of FA

The kinetics of FA- and Aedes-AT-stimulated activities of CA complexes were different (Figs 1, 2); FA-stimulated rates of JH III biosynthesis exceeded by far those of Aedes-AT-stimulated rates. MF increases were more elevated in FA-stimulated CA complexes than in FA + Aedes-AT-stimulated CA. MF increases were not observed in the Aedes-AT-stimulated CA. These results suggest that the activity of methyl farnesoate epoxidase is lower than that of *O*-methyl transferase in FA-stimulated CA complexes. One possible explanation for these changes in MF and JH III concentrations is that methyl farnesoate epoxidase was more activated than *O*-methyl transferase when stimulated by Aedes-AT in the presence of FA, while both enzymes (*O*-methyl transferase and methyl farnesoate epoxidase) were equally stimulated by Aedes-AT in the absence of FA.

With the exception of CA from newly emerged females, when CA complexes were incubated with Aedes-AT in the presence of FA, there were no additional increases in the rates of JH III biosynthesis, suggesting that Aedes-AT is involved in activating some rate-limiting step(s) preceding FA biosynthesis. Thus, when FA is added to the incubated CA, these rate-limiting steps are avoided, and synthesis of JH III is exclusively dependent on methyl-transfer and epoxidation. The same results have been found in FA- and allatotropin-stimulated CA of adult female *L. migratoria* (Gadot and Applebaum, 1986). In newly emerged virgin female *A. aegypti*, Aedes-AT had the ability to stimulate CA complexes to synthesize JH III only in the presence of FA. These data suggest the existence of an additional mechanism of CA stimulation in newly emerged females. Specific inhibitors for *O*-methyl transferase and methyl farnesoate epoxidase might be useful to elucidate the different stimulation pathways used by FA and Aedes-AT.

In summary, these studies demonstrate that Aedes-AT is a true allatotropin, which shows a dose–response relationship and age-specific effects, in *A. aegypti*. After the original description published by Kataoka et al. (1989), this is the first report confirming the CA-stimulatory activity of an allatotropin that is different from Mas-AT. These studies also reveal that FA stimulated JH biosynthesis and caused MF increases, while Aedes-AT did not, indicating that these two regulators differentially modulate the activity of the enzymes involved in the terminal steps of JH biosynthesis.

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