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Inhibition of Male Corpora Allata Activity and Sexual Pheromone Responsiveness in the Black Cutworm, *Agrotis ipsilon*, by the Hypocholesterolemic Agent, Fluvastatin

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Male sexual behavior of the black cutworm *Agrotis ipsilon* is controlled by corpora allata. Allatectomies performed on day-1 and day-3 males inhibited the typical sexual behavior in day-4 males when exposed to female pheromone in a wind tunnel. Both JH-III and JH-III acid were able to restore male sexual behavior, suggesting that corpora allata act through their endocrine activity. We demonstrated that corpora allata incubated in vitro produced only the acid form of JH-III and JH-II. Fluvastatin, an HMG-CoA reductase inhibitor, inhibited JH acid biosynthesis by the corpora allata when fluvastatin was added to incubation medium or injected in males 4 h before the bioassay. However, endocrine activity of CA resumed 5–6 h after injection, indicating that the effect of fluvastatin was temporary. Fluvastatin injection also induced temporary inhibition of male responsiveness.

Key words: insect, *Agrotis ipsilon*, juvenile hormone biosynthesis, corpora allata, male sexual behavior, pheromone responsiveness, fluvastatin, Hydroxymethylglutaryl-CoA reductase, cholesterol

INTRODUCTION

In most Lepidoptera, males respond to a sex pheromone produced in a gland on the abdominal tip of the conspecific females. Reception of the pheromonal blend by male antennae leads to sexual behavior and mating. In most species examined, sex pheromone production by the female is controlled by a Pheromone Biosynthesis Activating Neuropeptide (PBAN*), originating in

*Abbreviations used: CA = corpora allata; JH I, II, III = juvenile hormone homologs; PBAN = pheromone biosynthesis activating neuropeptide; SOG = subesophageal ganglion.

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the suboesophageal ganglion (SOG) (Raina and Klun, 1984). Although PBAN is the main factor involved in pheromone biosynthesis, recent studies have shown that juvenile hormone (JH), produced in the corpora allata (CA), regulates the female sexual behavior (pheromone production and release) of two noctuid moths, *Pseudaletia unipuncta* (Cusson and McNeil, 1989) and *Agrotis ipsilon* (Gadenne, 1993; Picimbon et al., 1995).

Moreover, CA of *A. ipsilon* seem to be also involved in pheromone responsiveness in males (Gadenne et al., 1993). Males deprived of CA did not exhibit their sexual behavior on day-4 when they were submitted to a pheromone blend in a wind tunnel. Unoperated males of *A. ipsilon* did not show any sexual pheromone responsiveness on the day of emergence but they efficiently responded to the pheromone after a few days. Since the primary olfactory system is functional as early as day-0 (Gadenne et al., 1993), the lack of responsiveness in young males may result from a required maturation process in which CA would be involved. In *Pseudaletia unipuncta*, JH acid biosynthesis by CA in vitro and male responsiveness to the female pheromone both increase with age under long day conditions, suggesting that CA may play a role in the induction of responsiveness, although this relationship is not so clear under short day conditions (Dumont and McNeil, 1992; Cusson et al., 1993, 1994). However, direct evidence that CA are involved in male responsiveness through the release of JH acid is lacking.

To clarify the role of JH in the pheromone responsiveness of male moths, we investigated the effects of JH deprivation as a function of time by performing early and late surgical allatectomies or by pharmacological inhibition of JH biosynthesis using fluvastatin, a potent HMG-CoA inhibitor active on insect CA (Debernard et al., 1994).

MATERIALS AND METHODS

Insects

Larvae of the black cutworm were reared on an artificial diet and maintained in individual plastic cups until pupation under 16L:8D at 21 ± 1°C. Pupae were observed each day for day-0 (newly emerged) adults. Adults were held in plastic boxes (two males in one box) and had access to 20% sucrose solution.

Chemicals

L-[methyl-^{3}H] Methionine and [^{3}H] JH-III were purchased from NEN (Boston, MA). [^{14}C] sodium acetate was purchased from Amersham International (Buckinghamshire, UK). JH-III and Ficoll were from Sigma (Taufkirschen, Germany). JH-II was purchased from SciTech., Prague, Czech Republic. Fluvastatin (Sandoz compound XU 62-320) was a gift from Dr. F. Kathawala (Sandoz Research Institute, Hanover, NJ). JH acid was obtained by enzymatic procedure, using pig liver carboxyesterase (Sigma). One micromole of JH acid is obtained from JH with 3 enzyme units (1 h, 40°C in phosphate buffer 50 mM, pH 7.4, yield >90%).

JH Biosynthesis

Rates of JH biosynthesis were determined in vitro using a radiochemical assay (Pratt and Tobe, 1974). Corpora allata were dissected and incubated for 4 h at 28°C in Medium TC 199 (75 µl, Flow Laboratories, Irvine, UK) supplemented with Ficoll (20 mg/ml), [2-^{14}C] sodium acetate (final concentration, 943 µM; specific activity, 1.08 Bq/pmol) and with L-[methyl-^{3}H] methionine (final concentration, 101 µM; specific activity, 17.27 Bq/pmol).

Rates of JH acid biosynthesis were calculated according to the following procedure. Female CA incubated in strictly similar conditions produce JH-III and JH-II dually labelled. One methyl group from methionine incorporated in 1 JH molecule. On the basis of this, acetate incorporation was found to be 0.9 and 0.8 labelled acetate incorporated into 1 JH-III and 1 JH-II molecules, respectively. We assumed that acetate incorporation into JH acid in males was the same.

In one series of experiments (in vivo effects of fluvastatin), corpora allata were from animals that had been injected with fluvastatin (30 µg), 3 or 5 h prior to dissection. Corpora allata were otherwise from untreated animals and fluvastatin (5 or 10 µM) was added directly to the culture medium as aqueous solution. After incubation, both medium and corpora allata were extracted with ethyl acetate (1 ml twice). The organic phase was evaporated under nitrogen flow and the sample injected in our RP-HPLC system.

Reverse Phase Liquid Chromatography (RPLC)

Analytical conditions were essentially those described by Halarnkar and Schooley (1990). The HPLC system (Beckman System Gold HPLC, Palo Alto, CA) with UV detector (Beckmann 166) is connected to a radioactivity monitor (Berthold LB 506, Postfach, Germany). The column is a 50 × 4.6 mm polymer column (PLRP-5, 5 mm, 100 A) protected with a 5 × 3 mm PLRP-5 guard cartridge (Polymer Laboratories, Amherst, MA). Buffer A was 5 mM Hepes adjusted to pH 6.2. Buffer B was 5 mM Hepes buffer (final concentration) in 80% acetonitrile. JH and its catabolites were separated at a flow rate of 1 ml/min using a linear gradient from 5 to 100% buffer B in 20 min. UV was monitored at 245 nm.

Corpora Allata Extirpations

For behavioral experiments, corpora allata extirpations (allatectomies) and sham-operations on male *A. ipsilon* were carried out at day-1 and at day-3 according to Gadenne et al. (1993). True allatectomy was verified by fine dissection of the head, once the behavioral test was finished.

Injection Procedures

Injections of either fluvastatin, JH-III or JH-III acid in males of *A. ipsilon* were carried out between the fourth and the fifth abdominal sternites using a 10 µl Hamilton-syringe. Injection volume was always 2 µl. Injection of JH-III or JH-III acid (5 µg) was performed in olive oil solution the day before the bioassays test to allow its diffusion into the moth. Controls were performed by injection of olive oil. Solubility of JH-III acid in olive oil was around 100%

when checked using labelled molecule. When injected into locusts, fluvastatin acts up to 5–6 h following injection (Debernard et al., 1994); the inhibitor was therefore injected, in *A. ipsilon* males, in water solution 3 and 5 h before the behavioral test. Sham injections were performed by injecting water in males.

Behavioral Analysis

Level of sex pheromone responsiveness of males of *A. ipsilon* was analysed by assessing their behavior in wind tunnel experiments (Gadenne et al., 1993). Pheromonal glands from mature day-4 females were excised at mid-scotophase. Pheromone was extracted by soaking the glands in hexane (50 µl equivalent for each gland) for 1 h. Experimental and control day-4 sexually mature males were exposed once to 1 female equivalent hexanic gland extracts, dispensed on a filter paper. Male behavior was observed for claspers extrusion, taking off and interrupted oriented flight, complete oriented flight, and landing on the source. Statistical analysis was performed using χ^2 test.

RESULTS

Involvement of CA and JH in Male Pheromone Responsiveness

Allatectomized males did not respond to a pheromonal gland extract when tested on day-4 in a wind tunnel (Table 1) but sexual behavior was restored when allatectomized individuals were injected with either JH-III or JH-III acid. JH-III acid seemed to be more efficient than JH-III in restoring behavior in allatectomized males, although the difference was not significant. When allatectomies were performed on day-3, males did not respond when tested in the wind tunnel on day-4. All together, these results suggest that corpora allata are involved in male receptivity behavior through their JH endocrine activity. Either JH or JH acid or intact corpora allata are required a few hours before behavioral test for optimal response.

Biosynthetic Activity of Male CA From *A. ipsilon*

In order to determine the nature of JH compounds produced by male CA, they were incubated for 4 h in incubation medium containing both labelled methionine and acetate. The typical radiochromatogram presented in Figure 1 shows that CA from day-4 adult males did not produce JH compounds, but only JH acid products. No radioactivity linked with a methylated form of JH could be detected (^{3}H line). On the contrary, JH-II acid (Rt = 13:18), JH-III acid (Rt = 11:40), and their corresponding JH acid diol were produced (Rt = 8:36 and 7:20, respectively) and identified by comparison with cold standards detected by their UV absorbance (245 nm). Additionally, 2 unidentified compounds called A and B (Rt = 10:24 and 11:12) were also detected.

Effects of Fluvastatin on CA Production

When male corpora allata were incubated in medium containing fluvastatin, the rate of JH acid biosynthesis was strongly inhibited (Fig. 2). A and B compounds were also inhibited, suggesting that they are some mevalonate derivatives (not shown).

TABLE 1. Effect of Allatectomy and Hormonal Rescue Therapy on Pheromone Responsiveness of Day-4 Males of *A. ipsilon

	C (%)	-CA D1 (%)	-CA D1 + OIL D3 (%)	-CA D1 + JH D3 (%)	-CA D1 + JHa D3 (%)	-CA D3 (%)
Number	23	29	16	10	14	19
No response	5 (22)	24 (83)	13 (81)	4 (40)	4 (29)	14 (74)
Claspers	9 (39)	4 (13)	3 (19)	5 (50)	5 (36)	4 (21)
Interrupted flight	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)
Complete flight	3 (13)	0 (0)	0 (0)	0 (0)	2 (14)	0 (0)
Landing	6 (26)	0 (0)	0 (0)	1 (10)	3 (21)	1 (5)

*C = control; -CA D1 = day-1 allatectomized males; + OIL D3 = injection of olive oil on day 3; + JH D3 = injection of JH-III on day 3; + JHa D3 = injection of JH-III acid on day 3; -CA D3 = day-3 allatectomized males.

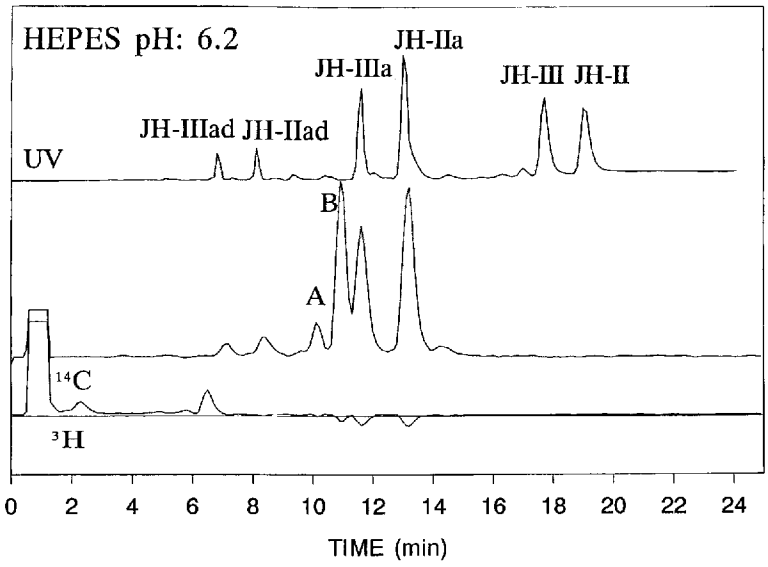

Fig. 1. Radio RPLC chromatograph of ethyl acetate extracts of incubation medium containing 3 pairs of CA of day-4 males of *A. ipsilon*. ^{3}H trace results from [methyl ^{3}H]-methionine incorporation, [^{14}C] trace results from [^{14}C] acetate incorporation. UV trace corresponds to co-injected standards monitored at 245 nm. Representative trace of more than 20 distinct radiochromatograms. **A** and **B:** unknown compounds, see text. a: acid; ad: acid diol.

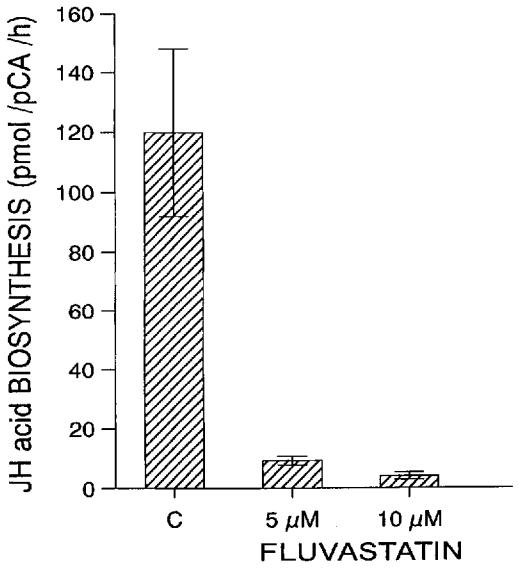

Fig. 2. Rate of JH acid biosynthesis (JH-II and JH-III acid) by corpora allata from day-4 males of *A. ipsilon* assayed in vitro in TC 199 supplemented with fluvastatin. Bars are the mean ± ESM for 5 individual determinations resulting from incubation of 3 p. CA each.

When fluvastatin (30 μg in aqueous solution) was injected into the abdomen of day-4 males 4 h before CA extirpation and incubation, JH acid biosynthesis was markedly inhibited (Fig. 3). However, if extirpation and incubation of the CA occurred 5 or 12 h post injection, there was partial resumption of JH acid biosynthesis. Fluvastatin thus inhibited in vitro and in vivo biosynthesis of JH product in the male CA although inhibition was transient when fluvastatin was injected in vivo.

Effects of Fluvastatin on Pheromone Responsiveness of the Males

Fluvastatin strongly inhibited pheromonal responsiveness of injected males when injected 3 h before the behavioral test as compared with the responsiveness of control and water-injected males (Table 2). However the effect of fluvastatin decreased with time, as the response of the tested males injected 5 h prior to behavioral test was not significantly different from control males (Table 2).

DISCUSSION

In *A. ipsilon*, CA are involved in the control of pheromone responsiveness (Gadenne et al., 1993). Using JH and JH acid injections into allatectomized males, we now show that CA act through their endocrine activity. However, although both JH-III and JH-III acid were able to restore sexual behavior in allatectomized males, we do not know about the nature of the JH molecule produced by the CA of *A. ipsilon*. Our in vitro incubation using both [^{3}H] methyl methionine and [^{14}C] acetate combined with RP-HPLC analyses clearly demonstrate that CA are producing JH-II acid and JH-III acid and their corresponding acid diols. These findings are in good agreement with the classi-

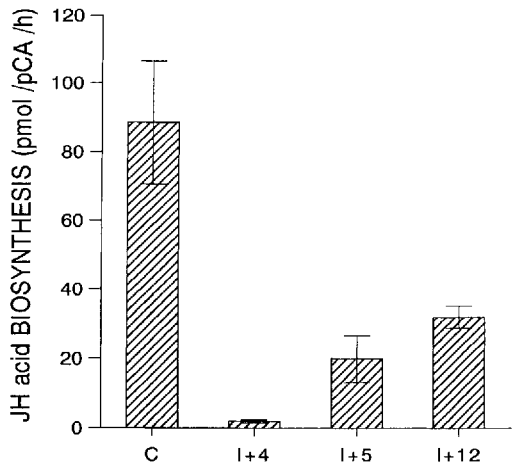

Fig. 3. Rate of JH acid biosynthesis (JH-II acid and JH-III acid) by corpora allata from day-4 males of *A. ipsilon* injected with 30 μg fluvastatin. CA were extirpated 3 h (I + 3), 5 h (I + 5), and 12 h (I + 12) following injection. Bars represent the mean rate of 5 individual determinations of 3 p. CA each.

TABLE 2. Effect of Fluvastatin on Pheromone Responsiveness of Day-4 Males of *A. ipsilon**

	Control (%)	Sham I+3 (%)	+Fluva I+5 (%)	+Fluva I+3 (%)
Number	23	23	25	26
No response	5 (22)	6 (26)	12 (48)	18 (69)
Claspers	9 (39)	13 (57)	5 (20)	8 (31)
Interrupted flight	0 (0)	1 (4)	2 (8)	0 (0)
Complete flight	3 (13)	0 (0)	0 (0)	0 (0)
Landing	6 (26)	3 (13)	6 (24)	0 (0)

*Males were tested 3 h (I+3) and 5 h (I+5) after injection of fluvastatin (30 µg). SHAM I+3 = day-4 males tested 3 h after injection of 2 µl distilled water.

cal scheme proposing that JH acid is released by CA of male moths due to the lack of JH acid methyl transferase (Peter et al., 1981; Bhaskaran et al., 1988; Cusson et al., 1993). It has been hypothesized that JH acid is released in the haemolymph and then stored in the accessory sex glands which have the capacity to methylate JH acids because they possess high juvenile hormone acid methyltransferase activity (Dahm et al., 1981; Shirk et al., 1983). However, we have currently no hypothesis that such a process occurs in *A. ipsilon*. Alternatively, JH acid could be the active form in the regulation of sexual behavior responsiveness and exogenous JH is converted into JH acid by hemolymphatic carboxyesterases. Additional experiments are required to clarify this question.

Also interesting is the production of unidentified compounds A and B released by the corpora allata in vitro. Both A and B are inhibited by fluvastatin suggesting that they are mevalonate derivatives. Work is in progress in our laboratory to identify these compounds in regard to recent and quite similar observations in *Pseudaletia unipuncta* (Cusson et al., 1991) and *Manduca sexta* (Granger et al., 1995).

Males allatectomized early in their adult life (day-1) did not respond to the female pheromone when they were submitted to a wind tunnel test. Complementation with exogenous hormone, 1 day before the wind tunnel assay, was sufficient to restore a normal behavioral response in males deprived of CA from day-1. If a maturation process regulated by JH is involved in male responsiveness, this process is resumed in one day after JH injection.

The antennal pheromonal perception system of allatectomized males is functional. Similarly, antennae from newly emerged males exhibit a normal sensitivity to the pheromone. However, both newly emerged and allatectomized males do not respond to the pheromone (Gadenne et al., 1993). JH effect could occur somewhere between the antennal level and the behavioral response. In the American cockroach, males of *Periplaneta americana* infected with the parasite *Moniliformis moniliformis* show a decreased response to sex pheromone although their peripheral level is not altered, thus suggesting that this inhibition of sexual behavior by parasites would occur at a central nervous system level (Carmichael et al., 1993). JH may act at the central nervous system as suggested for other behaviors in the honey bee (Withers et al., 1993) and in the house cricket (Cayre et al., 1994).

In contrast, allatectomy performed on day-3 suppressed male responsive-

ness on day-4 showing that the presence of CA during the first 3 days was not able to confer responsiveness the next day following allatectomy. All together these data suggest that production of JH or JH acid by the corpora allata is necessary at a time close to the behavioral assay. Similar results were obtained in *Pseudaletia unipuncta* females in which production of pheromone is controlled by juvenile hormone which must be present, even after the behavior has been initiated (Cusson et al., 1994). On the contrary, in males of *P. unipuncta*, JH acid or JH would act as a primer in the activation of responsiveness to the sex pheromone (Cusson et al., 1993). JH could also exert a primer effect on oviposition behavior in *Acheta domesticus* (Renucci et al., 1992).

Fluvastatin, added in incubation medium, inhibited JH acid production of male CA. In vitro inhibition of JH biosynthesis was also obtained with other HMG CoA reductase inhibitors such as compactin (Monger et al., 1982; Edwards and Price, 1983; Belles et al., 1988) or mevinolin (Feyereisen and Farnsworth, 1976; Couillaud, 1991). The rate of JH production was also inhibited when fluvastatin was injected into the moth up to 4 h before CA extirpation. However, when the delay between the time of injection and of CA extirpation increased, CA gradually escaped from the fluvastatin inhibition as reported in the locust (Debernard et al., 1994). This transient inhibition of JH production following fluvastatin injection was used to investigate the effect of JH production deficiency in the moth at the time of wind tunnel assay. Clearly, such inhibition could not be the result of allatectomy associated surgical trauma.

Moreover, we demonstrate that male responsiveness was inhibited by fluvastatin and that the duration of this inhibition was limited to the period of total inhibition of JH production. In vivo anti-JH effects of HMG-CoA reductase inhibitor are limited. Repeated injections of compactin induced black pigmentation in *Manduca sexta* larvae (Monger et al., 1982) and repeated injections of fluvastatin resulted in lengthening of the larval stadium in *Locusta migratoria* (Debernard et al., 1994). Present report is the first demonstration that HMG-CoA reductase inhibitor may have a strong behavioral effect in an insect.

This paper demonstrates that corpora allata are involved in responsiveness in males *Agrotis ipsilon* through their JH acid endocrine activity. JH acid production by the CA is necessary at the precise time of behavioral performance. However, nothing is known about the exact mechanism of this endocrine regulation of male sexual behavior.

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