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Stimulation of Sex Pheromone Production by PBAN-Like Substance in the Pine Caterpillar Moth, *Dendrolimus punctatus* (Lepidoptera: Lasiocampidae)

Cheng-Hua Zhao,* Qun Li, and Wei Gao

Sex pheromone production in the female pine caterpillar moth, *Dendrolimus punctatus* is controlled by a PBAN-like substance located in the head of female moth. Pheromone titer was significantly decreased by decapitation of female moth, and restored by injection of either Hez-PBAN or head extract prepared from male or female moth. Stimulation of pheromone production by head extract followed a dose-dependent pattern from 0.5 to at least 4 head equivalent. A gland in vitro assay was used to study the relationship between gland incubation time and pheromone production as well as calcium involvement in the stimulation of pheromone production by head extract. Maximum pheromone production was occurred at 60 min after pheromone gland was incubated with two equivalents of head extracts. In vitro experiments showed that the presence of calcium in the incubation medium was necessary for stimulation of pheromone production. The calcium ionophore, A 23187, alone stimulated pheromone production. The pheromone components (*Z,E*)-5,7-dodecadienol and its acetate and propionate were produced in these experiments but in addition to the aldehyde, (*Z,E*)-5,7-dodecadienal was also found. This indicates that females are capable of producing four oxygenated functional groups. The PBAN-like substance control of the pheromone biosynthetic pathway was investigated by monitoring the incorporation of the labeled precursor into both pheromone and pheromone intermediates. Arch. Insect Biochem. Physiol. 49:137–148, 2002.

KEYWORDS: PBAN-like substance; pheromone production; calcium; A23187; *Dendrolimus punctatus*

INTRODUCTION

Production of female sex pheromone in many moth species is controlled by the pheromone biosynthesis activating neuropeptide (PBAN) produced in the brain-subesophageal ganglia complex. This peptide, consisting of 33 amino acids, was first isolated and sequenced from *Helicoverpa zea* and termed Hez-PBAN (Raina et al., 1989). PBAN has since been isolated from a number of moth species and has been shown to stimulate pheromone production in many moths.

The sex pheromone of *Dendrolimus punctatus* (Lasiocampidae) consists of three major pheromone components, Z5,E7-12:OH, Z5,E7-12:OAc, and Z5,E7-12:OPr. The ratio of the pheromone components was found to be highly variable between individual females, but Z5,E7-12:OH was often the most abundant component (Laboratory of Insect Pheromone, 1979; Zhao et al., 1993).

Abbreviations used: DMSO = dimethyl sulfoxide; GC-MS = gas chromatography-mass spectrometer; ME = male head extract equivalent; PBAN = pheromone biosynthesis activating neuropeptide. Pheromone compounds and analogs are abbreviated in following way with (in order) geometry of the double bond (Z or E), position of double bond (number), chain length (number), and functionality. For example, Z5,E7-12:COOH = (*Z,E*)-5,7-dodecadienic acid; D_4-Z11-18:COOH = [15,15,16,16-$^2H_4$]-(*Z*)-11-octadecenoic acid; Z5,E7-12:OH = (*Z,E*)-5,7-dodecadienol; Z5,E7-12:OAc = (*Z,E*)-5,7-dodecadienyl acetate; Z5,E7-12:OPr = (*Z,E*)-5,7-dodecadienyl propionate; Z5,E7-12:Me = (*Z,E*)-5,7-methyl dodecadienoate.

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Attraction of the conspecific male and mating normally occur during the last two hours of the scotophase (Section of Insect Pheromone, 1973). This implies that pheromone titers of *D. punctatus* fluctuate diurnally and the fluctuation in pheromone titer could be controlled by a brain factor that may be similar to PBAN in *H. zea* (Raina and Klun, 1984; Raina et al., 1989). In the present study, we investigated whether a PBAN-like substance could control pheromone production, how this substance controls pheromone biosynthesis, as well as calcium involvement in the stimulation of pheromone production.

MATERIALS AND METHODS

Chemicals

Synthetic Hez-PBAN was purchased from Peninsula Laboratories (Belmont, CA). Sterile TC 199 insect medium was prepared from powder form that was purchased from Difco Laboratories (Detroit, MI). The calcium ionophore A23187 was obtained from Sigma Chemical Company (St. Louis, MO). D_4-Z11-18:COOH (the deuterium enrichment was 93.5%) was a gift from Richard Adlof. All of reference compounds, including pheromone components, were available in our laboratory.

Insect Source, Preparation of Head Extracts, and Bioassay

Pupae were collected from Jiangxi Province of China and kept at 16:8 L:D cycle and room temperature. The average amount of the most abundant pheromone component, Z5,E7-12:OH in normal females of *D. punctatus*, varied from one generation to another. Therefore, the insects used in any single experiment were limited within one collection. After emergence, males and females were separated and maintained at the same condition. All insects used for head extracts were taken during the photophase approximately 1–2 h before light off, unless otherwise noted. Severed heads of 1–2-day-old male or female moths, with antennae and palpi removed, were placed in cold saline (NaCl, 188.0 mM; KCl, 20.1 mM; $CaCl_2$, 9.0 mM; $MgSO_4$, 1.0 mM) and homogenized with a glass homogenitor for 2 min. The homogenites were centrifuged at 25,000*g* and 4°C for 10 min. The supernatant was diluted to 0.1 head equivalents/μl and then stored at –20°C until assayed. Females (1–2 days old) used for both in vivo and gland in vitro assay were decapitated 1 h before scotophase, and then kept in an incubator for 24 h. The indicated amount of head extracts or synthetic Hez-PBAN dissolved in 20 μl of saline or saline only was injected into the abdomen of each decapitated female and then incubated for 1 h.

For gland in vitro assay, the intersegmental membrane containing pheromone-producing cells between the eighth and ninth abdominal segments was dissected from decapitated females and immediately placed in sterile TC 199 or saline medium. All internal tissues attached to the pheromone gland were carefully removed and the remaining cylinder of epidermis and cuticle was washed 2–3 times with the medium. The cylinder was then cut open to allow the pheromone gland to float on top of the medium with the cellular side down exposed to the medium. Two isolated pheromone glands were incubated with or without test material in each drop of incubation medium for 1 h at room temperature, unless otherwise noted.

After incubation, a given number of pheromone glands were removed from either decapitated females or incubation medium and extracted with hexane containing 10 ng Z9-14:OAc as an internal standard.

Labeling Experiments

Females were either kept intact (normal group) or decapitated at 1 h before darkness. The two groups were then maintained at a 16:8 L:D cycle for 24 h. Pheromone glands were extruded with plastic clips, and then 10 μg of D_4-Z11-18:COOH in 0.4 μl DMSO was topically applied to each gland at 1 h before darkness. Fifty minutes later, the insects were released into a cage and kept in the darkness for approximately 7 h. A given number of

glands were then dissected and extracted for 24 h with 20 µl of chloroform/methanol (v/v = 2:1) containing 50 ng of Δ13–14:Me and 15:OH as internal standards. Base methanolysis was performed to convert fatty acyl moieties to the corresponding methyl esters and pheromone esters to the pheromone alcohol for GC-MS analysis as described by Zhao and Wang (1990).

Analytical Methods

GC analyses were conducted with a Pye Unicam series 204 chromatograph equipped with a splitless injector and a flame ionization detector. Hydrogen flow rate was 50 cm/s and the purge valve was opened 0.25 min after injection. The following capillary columns (SGE, Australia) were used: (A) 25 m × 0.22 mm i.d. BPX-70, (B) 25 m × 0.22 mm i.d. BP-20, (C) 25 m × 0.22 mm i.d. BP-5. The temperature program for all columns was used as follow: 1 min at 80°C and then 3°/min to 200°C, held for 20 min.

A Finnigan ITD 800 mass spectrometer in EI mode interfaced to a HP 5890A gas chromatograph was used for analyses of the gland extracts. For monitoring the incorporation of D_4-Z11-18:COOH into Z5,E7-12:OH and the pheromone intermediates, a Finnigan Voyager mass spectrometer in EI mode interfaced with a Trace 2000 gas chromatography was used. A BP-20 column (25 m × 0.22 mm i.d) was used in both GC-MS systems. Column temperature was maintained at 80°C for 1 min, then raised at a rate of 4°/min to 200°C and held for 20 min. Injection was made in the splitless mode and the purge valve was opened 0.75 min after injection. The single ion monitoring mode was used to detect following diagnostic ions: 164 and 168 for unlabeled and D_4-labeled Z5,E7-12:OH respectively; 236 and 240 for unlabeled and D_4-labeled Z9-16:Me; 238 and 242 for unlabeled and D_4-labeled Z7,E9-14:Me; 210 and 214 for unlabeled and D_4-labeled *Z*5,E7-12:Me; 208 and 210 for internal standard, Δ13-14:Me (for quantification of the pheromone intermediates as methyl esters) and 15:OH (for quantification of Z5,E7-12:OH).

Statistical Analysis

Statistical analysis was performed by ANOVA in all of the figures. Differences among means were tested for significance by the Newman Keuls test at $P < 0.05$. The unpaired two-tailed t-test was used to compare two or three means in Tables 1 and 2.

RESULTS

Effect of PBAN and Head Extract on Pheromone Production in Decapitated Females

Decapitation of females for 24 h resulted in a decrease in the titer of Z5, E7-12:OH from 3.5 ± 2.1 ng to 0.3 ± 0.2 ng. Injection of 10 pmol Hez-PBAN into decapitated female resulted in a significant increase in the titer of Z5,E7-12:OH.

TABLE 1. Effect of Head Extract, Hez-PBAN, and Calcium Ionophore A 23187 on Production of Pheromone Components and Related Compound of *D. punctatus* After In Vivo or Gland In Vitro Incubation*

	Titer of pheromone or pheromone-related compound (ng/gland)			
Pheromone source	Z5, E7-12:OH	Z5, E7-12:OAc	Z5, E7-12:OPr	Z5, E7-12:Ald
In vivo experiment				
Normal female	3.2 ± 1.6[a]	0.6 ± 1.0[a]	1.1 ± 0.7[a]	—
Decapitated female + head extract	7.3 ± 3.7[b]	4.9 ± 2.6[b]	1.9 ± 0.6[b]	1.1 ± 0.0[a]
Decapitated female + Hez-PBAN	2.1 ± 0.7[c]	5.4 ± 2.6[b]	1.8 ± 0.0	0.9 ± 0.1[a]
In vitro experiment				
Isolated gland + TC199	0.7 ± 0.3[A]	—	—	—
Isolated gland + head extract	29.1 ± 16.0[B]	1.0 ± 0.6[A]	1.3 ± 0.9[A]	7.7 ± 6.6[A]
Isolated gland + A23187	3.4 ± 2.0[C]	0.6 ± 0.5[A]	0.4 ± 0.2[B]	0.6 ± 0.8[A]

*For in vivo experiment (N = 4–6), 3–5 FE gland extracts were used for one replicate. For in vitro experiment (N = 5), 2 gland extracts were used for one replicate. Means followed by different letters within in vivo (lower case superscript letters) or in vitro (capital superscript letters) experiment, respectively, are significantly different at $P < 0.05$ (*t*-test).

[b]Z5, E7=12:OPr was only detected once in Decapitated female + Hez=PBAN.

Likewise, Z5, E7-12:OH titer of decapitated females injected with male or female head extracts was significantly higher than that of normal females or decapitated females injected with PBAN. No significant differences were found between decapitated females injected with male and female head extracts (Fig. 1). When decapitated females were injected with head extracts prepared from males taken during photophase or scotophase, Z5, E7-12:OH titers in two female groups were significantly increased to a much higher level compared with decapitated females only injected with saline. No significant difference was found between pheromonotropic activities of head extracts prepared from males taking during photophase and scotophase (Fig. 1).

In addition to three pheromone components Z5,E7-12:OH, Z5,E7-12:OAc, and Z5,E7-12:OPr, a gland component with the same retention time to Z5,E7-12:Ald on both BP-20 and BP-5 capillary column was found in the extracts of the decapitated female injected with head extracts. However, the aldehyde was not found in pheromone glands of normal females (Table 1). These results were confirmed by GC-MS analyses, which showed that a peak with the same retention time to Z5,E7-12:Ald produced a mass spectrum that was identical to synthetic Z5,E7-12:Ald (data not shown).

In Vitro Stimulation of Sex Pheromone Production by Head Extract

Incubation of isolated pheromone glands in TC 199 medium with head extracts resulted in a significant increase of pheromone production (Table 1). The acetate and propionate esters were increased to a level similar to that found in normal females, but Z5,E7-12:OH was increased to the highest observed in this study. Similarly, a relatively small amount of Z5,E7-12:Ald was found in decapitated females injected with head extracts, but a much higher amount of Z5,E7-12:Ald was found in the isolated pheromone glands incubated with head extracts.

Dose Effect and Time Response to Head Extract

The production of Z5,E7-12:OH followed a dose-dependent pattern after injection of decapitated females with various concentrations of male head extracts (Fig. 2). Although the lower doses

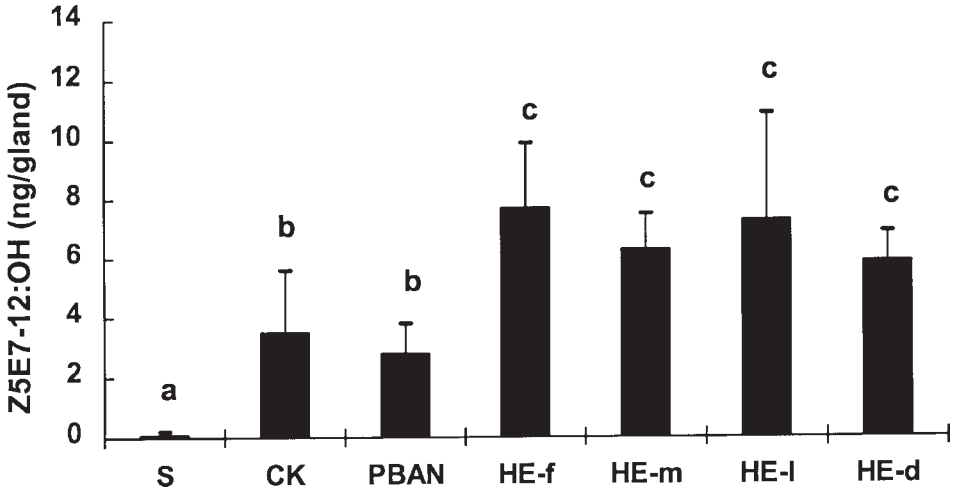

Fig. 1. PBAN-like substance effect on pheromone titers of decapitated females injected with saline alone (s) or saline containing 10 pmol Hez-PBAN (PBAN), head extracts from females (HE-f) or males (HE-m), head extracts of males taken from last 1–2 h of photophase (HE-l) or last 1–2 h of scotophase (HE-d). CK represents Z5,E7-12:OH titer in normal females. Dosage of head extracts used in all treatments was 2 equivalents/female. Bars represent the mean ± S.E. (4–6 replicates, 3 females/replicate). Bars with different letters are significantly different (Newman Keuls test, $P < 0.05$).

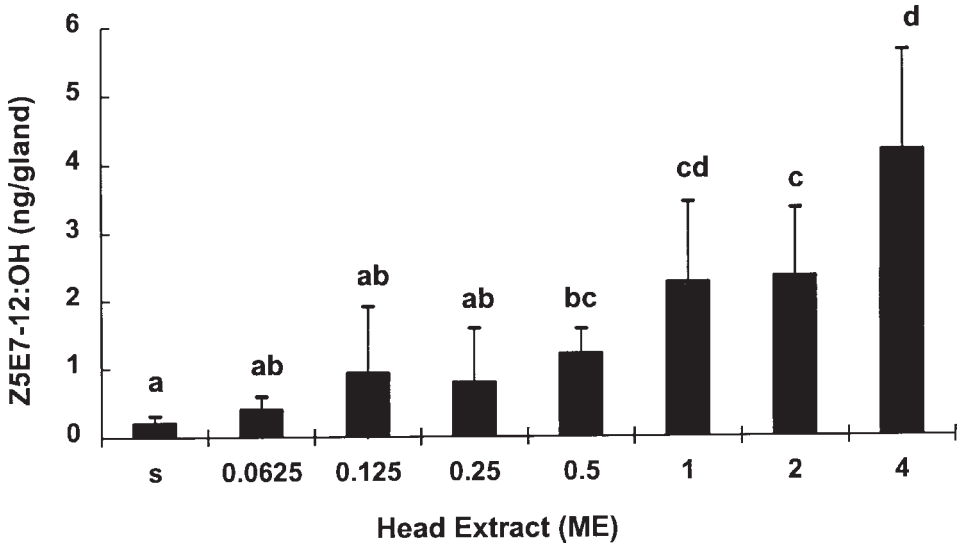

Fig. 2. Pheromonotropic response to different dosages of male extracts injected in decapitated females. "s" represents decapitated females injected with saline alone. Bars represent the mean ± S.E. of 3–6 replicates (3 females/replicate). Bars with different letters are significantly different (Newman Keuls test, $P < 0.05$).

produced higher levels of pheromone, a significant increase was found beginning at the 0.5 ME dose. The maximum response was observed at a dose of 4 ME. Other pheromone components, Z5,E7-12:OAc and Z5,E7-12:OPr followed the same pattern as Z5,E7-12:OH, with the maximum response of Z5,E7-12:OAc (2.79 ± 1.60 ng/gland) and Z5,E7-12:OPr (1.41 ± 0.62 ng/gland) occurring at a dose of 4 ME. However, Z5,E7-12:Ald did not follow the same pattern as pheromone components. The production of Z5,E7-12:Ald reached the maximum level at a dose of 2 ME, but higher dose did not increase the production (data not shown).

Isolated pheromone glands were incubated in the presence of 2 ME for various time periods. After 30-min incubation, the production of Z5,E7-12:OH was significantly increased (Fig. 3). Maximum stimulation of Z5,E7-12:OH production was found in the isolated glands that were incubated for 120 min (7.4 ± 3.4 ng/gland), but this stimulation was not significantly different from that incubated for 60 min (6.4 ± 1.7 ng/gland).

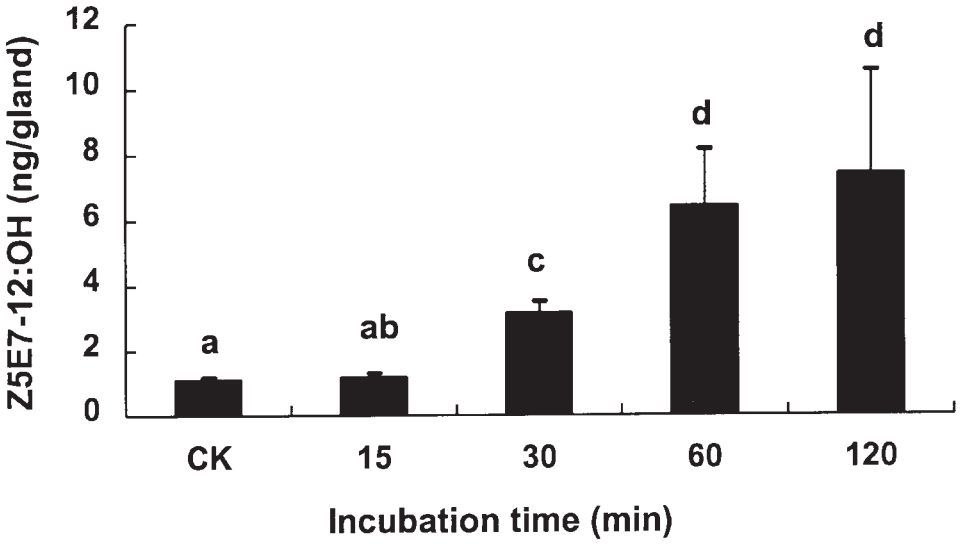

Fig. 3. Time-course of Z5,E7-12:OH titer in isolated glands incubated with or without (CK) 2 equivalents of head extracts. Each bar represents the mean ± S.E. of 3–4 replicates (one gland/replicate). Bars with different letters are significantly different (Newman Keuls test, $P < 0.05$).

Effect of Calcium on Pheromone Production Stimulated by Head Extract

Various concentrations of calcium ion in saline were prepared to test the in vitro effect of calcium on Z5,E7-12:OH production stimulated by head extract. The head extract was inactive when glands incubated in saline without calcium. A level of 0.1 mM calcium was the lowest concentration to significantly stimulate Z5,E7-12:OH production (Fig. 4). When calcium concentration was increased from 0.1 to 3 mM, Z5,E7-12:OH production gradually increased and reached the maximum amount at a calcium concentration of 3 mM. A higher concentration of calcium (10 mM) in the saline did not significantly increase Z5,E7-12:OH production.

A calcium ionophore, A23187, was used to test if increasing calcium inside pheromone gland cells would stimulate pheromone production. When isolated glands were incubated with the calcium ionophore, A23187, Z5,E7-12:OH titer was significantly increased to a level similar to normal females but significantly lower than that in isolated glands incubated with head extracts (Table 1). Furthermore, a dose response was obtained with various concentrations of A23187 (Fig. 5). Maximum stimulation of Z5,E7-12:OH production (9.6 ± 6.3 ng) occurred in the incubation with 100 μM A23187, and a higher dosage of A23187 did not result in a higher response.

Incorporation of Labeled Pheromone Precursor

To determine which step(s) in the pheromone biosynthetic pathway is controlled by PBAN, the incorporation of the labeled pheromone precursor, D_4-Z11-18:COOH, into the pheromone component Z5,E7-12:OH and its intermediates was monitored. No significant differences in titers of labeled Z5,E7-12:OH and intermediates, Z9-16:Me and Z5,E7-12:Me, were observed between normal and decapitated females, though unlabeled Z5,E7-12:OH titer of decapitated females was significantly

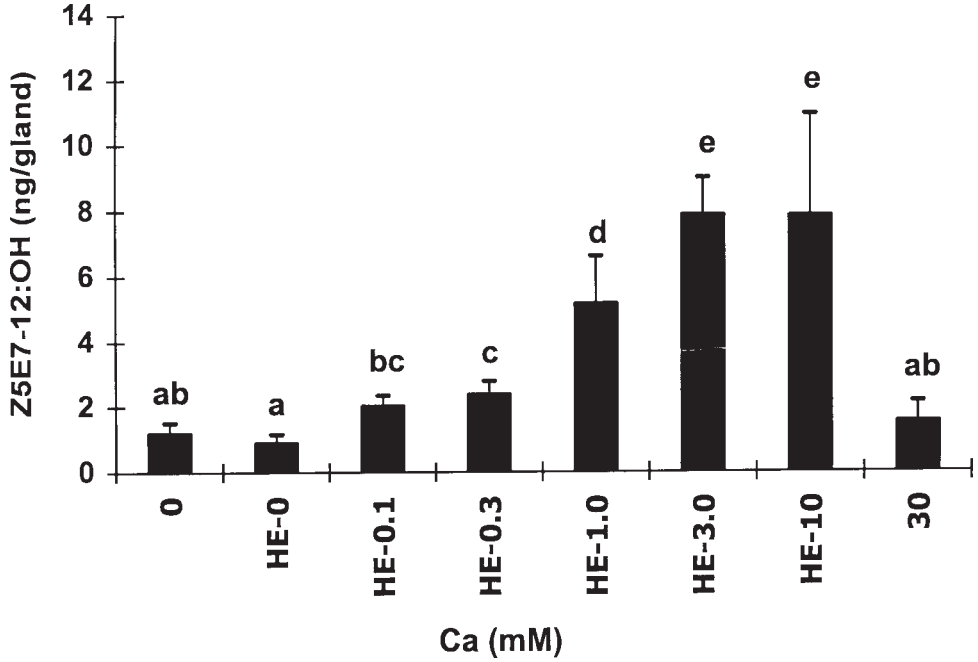

Fig. 4. Effect of calcium ion on PBAN-stimulated pheromone production. Pheromone glands were incubated with (HE) or without (0 and 30) 2 ME head extracts. Various concentrations (mM) of calcium ion are indicated at the bottom. The incubation media in HE-0 contained head extracts but not calcium ion (1 mM EGTA was added in the medium). The incubation media in 0 contained neither calcium ion nor head extracts. Bars represent the mean ± S.E. of 3–6 replicates (one gland/replicate). Bars with different letters are significantly different (Newman Keuls test, $P < 0.05$).

TABLE 2. Incorporation of D_4-Z11-18:COOH Into Pheromone and Fatty Acid Intermediates, Analyzed as Methyl Esters in Pheromone Gland of *D. punctatus*[†]

	Amount (ng/gland ± SEM, N = 4)			
	Normal female		Decapitated female	
Compounds	Unlabeled	D_4-labeled	Unlabeled	D_4-labeled
Z9-16:Me	80 ± 12	4.8 ± 2.4	60 ± 5.5*	3.3 ± 0.9
Z7,E9-14:Me	0.30 ± 0.10	—	0.33 ± 0.09	—
Z5,E7-12:Me	170 ± 27	5.0 ± 0.24	145 ± 197	3.0 ± 1.8
Z5,E7-12:OH	3.9 ± 1.8	0.28 ± 0.15	0.89 ± 0.27*	0.15 ± 0.06

[†]D_4–Z11–18:Acid was applied to normal (4 females/repicate) and decapitated females (8 females/replicate) as described in Materials and Methods. One of the pheromone intermediates, Z9E11–16:Me is not detectable (Zhao et al., unpublished data). —, below the limit of detection.
*Denotes significant difference from normal female values (*t*-test, $P < 0.05$).

decreased compared with that of normal ones (Table 2). It should be motioned that unlike other moth species, the incorporation of labeled fatty acid precursors into Z5,E7-12:OH in *D. punctatus* needs a rather long incubation time (8 h for labeled Z11-18:COOH; 18 h for labeled palmitic acid and Z9-16:COOH; Zhao et al., unpublished data). Therefore, a group of normal females instead of decapitated ones injected with PBAN were used in this experiment to keep the presence of PBAN for a relatively long time.

DISCUSSION

Decapitation of female *D. punctatus* in photophase resulted in a decline in the sex pheromone to a very low level in the subsequent scotophase. Pheromone production of a decapitated female could be restored by injection of synthetic Hez-PBAN or head extracts into the abdomen. However, the amount of Z5,E7-12:OH produced by the decapitated female injected with 10 pmol synthetic Hez-PBAN was significantly lower than that injected with 2 equivalents of female or male head extracts. The above results suggest that sex pheromone production in *D. punctatus* is under the control of a PBAN-like substance in the female head. Higher Z5,E7-12:OH titer in the decapitated females injected by female or male head extracts could be due to the use of a higher dosage of head extracts (2 ME). Another possibility as to why Hez-PBAN did not stimulate to the same extent as the

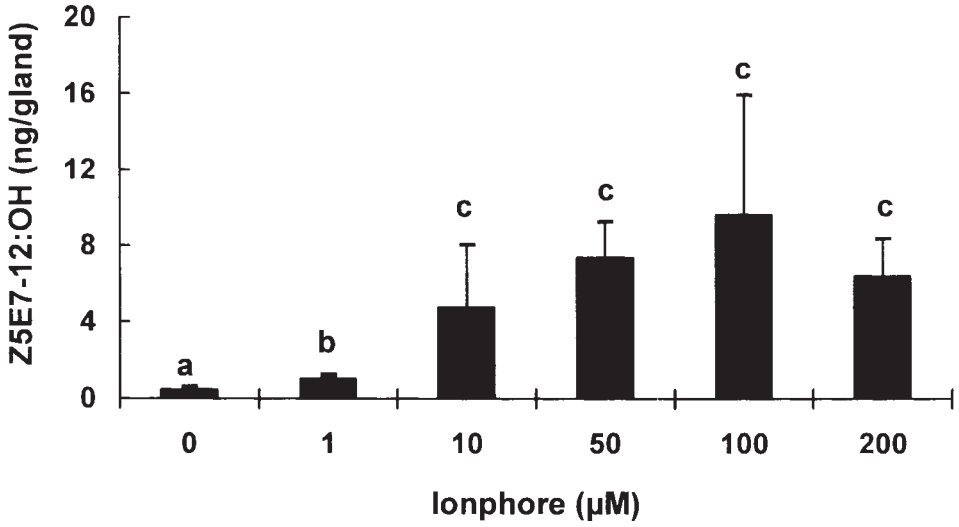

Fig. 5. Stimulation of pheromone production by the calcium ionophore, A 23187. Pheromone glands were incubated with various concentrations of ionophore in the saline containing 9 mM calcium ion. Each bar represents the mean ± S.E. of 3–4 replicates (one gland/replicate). Bars with different letters are significantly different (Newman Keuls test, $P < 0.05$).

head extract is that the normal PBAN found in *D. punctatus* has a different sequence and, thus, would be more active than the Hez-PBAN. The fact that the pheromontropic activity was found in the head of both male and female and during both photophase and scotophase is in agreement with many earlier reports (Martinez and Camps, 1988; Ando et al., 1988; Gazit et al., 1992; Altstein et al., 1993; Zhu et al., 1995).

In decapitated females, stimulation of Z5,E7-12:OH titer by the head extract followed a manner in which two steps of a linear dose-response were involved (no increase was found between dosages of 1 and 2 ME, see Fig. 2). This type of dose-response was similar to that found in *H. zea* response to synthetic Hez-PBAN (Raina et al., 1989) and Lyd-PBAN (Masler et al., 1994). A relatively wide linear relationship between dose and response (from 0.0625 to at least 4 ME) was found in *D. punctatus*. This is comparable to other species, for example, *Agrotis segetum*, in which maximum response was given with the injection of one-half brain-SOG homogenate (Zhu et al., 1995). We found twice as much Z5,E7-12:OH produced in decapitated females injected with two head extract equivalents compared to normal females (Fig. 1). The variability in pheromone production in this species is quite high and the utilization of a higher concentration of head extract may have ensured maximum pheromone production.

In the gland in vitro experiments, Z5,E7-12:OH titer was stimulated to a level, that was about 9 times higher than that in normal females or about 4 times higher than decapitated females injected with head extracts. However, the other pheromone component, Z5,E7-12:OAc and Z5,E7-12:OPr, were only stimulated to a level similar to that in normal females (Table 1). A large amount of the immediate pheromone precursor, Z5,E7-12:COOH, has been found in the pheromone gland of *D. punctatus* (Table 2). This fact implies that in TC 199, while Z5,E7-12:OH was converted from Z5,E7-12:COOH by reduction in a high conversion rate, Z5,E7-12:OAc and Z5,E7-12:OPr were converted from Z5,E7-12:OH by acetylation and propionation, respectively, only in a conversion rate as that in normal females. In contrast with the above results, the gland in vitro experiments using saline as a medium produced a different ratio of Z5,E7-12:OH to Z5,E7-12:OAc or Z5,E7-12:OPr, and this ratio was close to that in normal female (data not shown). Incubation medium influence of pheromone production in isolated gland stimulated by PBAN has been found in *Ostrinia nubilalis*, in which the incubated gland gives a consistent response only with Excell 401, but not with other media, Grace's insect medium, IPL-41, and modified lepidopteran saline (Ma and Roelofs, 1995).

A new gland component, Z5,E7-12:Ald, was found in decapitated females injected with head extracts or Hez-PBAN and isolated glands incubated with head extracts or a calcium ionophore, A23187, but not in normal females (Table 1). Z5,E7-12:Ald is a major pheromone component of a closely related species, *D. pini* (Prisner et al., 1984) and a likely inhibitor of the attractant pheromone blend in *D. punctatus* (Zhao, unpublished data). It may be hypothesized that decapitated females or gland incubations lose a factor or mechanism to control the specific pheromone blend produced by normal females. Losing this factor or mechanism caused an increased activity of an alcohol oxidase resulting in production of Z5,E7-12:Ald. The fact that an aldehyde together with corresponding alcohol was found in decapitated females or gland incubations stimulated by head extracts, suggested that an alcohol oxidase exists in the gland as in *Heliothis* moths (Teal and Tumlinson 1986, 1988). We also noted that in both in vivo and gland in vitro incubation as the Z5,E7-12:OH titer increased, Z5,E7-12:Ald titer increased accordingly. Therefore, it is conceivable that Z5,E7-12:Ald is a metabolic intermediate, when excess Z5,E7-12:OH needs to be removed by alcohol oxidation and subsequently aldehyde oxidation to corresponding acid, Z5,E7-12:COOH. An oxidase that converts primary alcohols to aldehydes and subsequently to fatty acids has been found on or in the cuticle of the sex pheromone gland and the papillae anales of *Manduca sexta* and a similar process has been suggested in the pheromone metabolism by Fang et al. (1995a).

It has been suggested that in *H. zea*, PBAN is synthesized in brain-suboesophageal ganglia, and then transported to corcora cardiaca. From there, the PBAN is released into the hemolymph, and then travels to the pheromone gland to stimulate pheromone production (Raina and Klun, 1984). This hypothesis was supported by various experiments including those that show pheromone production could be stimulated in isolated gland incubation with PBAN in several species, such as *H. armigera, H. zea, Bombyx mori, Spodoptera litura, S. littoralis, O. nubilalis,* and *Thaumetopoea pityocampa* (Soroker and Rafaeli 1989; Rafaeli et al., 1990; Jurenka et al., 1991a; Arima et al., 1991; Fonagy et al., 1992a; Fabrias et al., 1994; Ma and Roelofs, 1995; Fabrias et al., 1995). In contrast to the first PBAN action mode, other studies suggested another action mode, that PBAN in *H. zea* and *H. virescens* is transported via the ventral nerve cord to the terminal abdominal ganglion, which releases a second messenger, octopamine to act on the pheromone gland (Teal et al., 1989; Christensen et al., 1991). In the second mode, the intact ventral nerve cord is necessary for normal PBAN stimulation of pheromone biosynthesis. It has been found that both PBAN and octopamine can stimulate pheromone biosynthesis in isolated abdomens lacking ventral nerve cord only when abdomens were treated at the onset of scotophase (Christensen et al., 1991). In our gland in vitro experiments, the pheromone glands were treated by head extracts in photophase and we found that pheromone production was stimulated to a level that was even much higher than that in normal female. These results corroborate the above studies with *H. armegera, H. zea, B. mori, S. litura, S. littoralis, O. nubilalis* and *T. pityocampa* showing that pheromone production stimulated by PBAN-like substance follows the first action mode in a wide range of species. However, a conclusive proof that PBAN acts directly on pheromone gland cells will come only after identification, characterization, and purification of PBAN receptors (Jurenka and Roelofs, 1993).

In the present study, the gland in vitro experiment showed that head extract was active only when glands were incubated in saline containing calcium. A pheromonotropic action similar to that of the head extract, was observed when the calcium ionophore, A 23187, was applied to isolated gland incubations in the presence of extracellular calcium. These observations corroborate the results from *H. zea, B. mori, S. litura, H. armigera*, and *O. nubilalis* (Jurenka et al., 1991b; Fonagy et al., 1992b; Matsumoto et al., 1995; Soroker and Rafaeli, 1995; Ma and Roelofs, 1995), indicating that calcium is a second messenger that is necessary for signal transduction. In a proposed model for PBAN stimulation of the pheromone biosynthetic pathway in *H. zea*, PBAN binds to a receptor on the cell membrane that, in an as yet unknown way, activates a plasma membrane calcium channel. Then the rise in intracellular calcium stimulates adenylate cyclase to produce cAMP, which then activates enzymes involved in the signal cascade to finally stimulate key enzymes in the pheromone biosynthetic pathway (Jurenka and Roelofs, 1993). In *H. zea*, cAMP analogs and forskolin can stimulate pheromone production in the absence of extracellar calcium (Jurenka, et al., 1991b). In *H. armigera*, the calcium ionophore, ionomycin, can mimic the pheromonotropic action of PBAN by causing a significant elevation of intracellular cAMP (Rafaeli, 1994; Rafaeli and Gileadi, 1995). However, cyclic nucleotides failed to mimic or potentiate the pheromonotropic action of Bom-PBAN on isolated *O. nubilalis* pheromone glands (Ma and Roelofs, 1995). In *B. mori*, similar results were observed, although forskolin did potentiate the action of Bom-PBAN (Fonagy et al., 1992b). Therefore, it is worthwhile to test if cAMP is necessary for the intracellular signal transduction of PBAN in *D. punctatus*.

In *D. punctatus*, the biosynthesis of Z5,E7-12:OH begins from the production of palmitic acid via fatty acid synthesis. Palmitic acid is then converted to stearic acid by chain elongation, followed by Δ11 desaturation to form Z11-18:COOH. This intermediate is chain shortened to Z9-16:COOH followed by another Δ11 desaturation to form Z9,E11-16:COOH, and Z5,E7-12:OH is finally produced by two cycles of chain shortening and re-

duction (Zhao et al., unpublished data). In our study, no significant difference was found in the production of labeled Z5,E7-12:OH between decapitated and normal females, suggesting that the PBAN-like substance does not affect the biosynthetic steps converting Z11-18:COOH to Z5,E7-12:OH, including any chain shortening, Δ11 desaturation of Z9-16:COOH and reduction. Thus, the PBAN-affecting biosynthetic step in *D. punctatus* could be either an early step from palmitic acid to Z11-18:COOH including chain elongation and Δ11 desaturation of stearic acid or an even earlier step(s) from acetate to palmitic acid. It has been suggested that PBAN can affect an enzymatic step(s) in or prior to fatty acid synthesis in some species (Tang et al., 1989; Jurenka et al., 1991; Jacquin et al., 1994; Zhao and Li, 1996). However, in other species, it can affect the reduction step from fatty acids to alcohols or aldehydes (Martinez et al., 1990; Arima et al., 1991; Fabrias et al., 1994,, 1995; Fang et al., 1995b). Furthermore, acetylation, the last step of the pheromone biosynthetic pathway in *Sesamia nonagrioides* was shown to be controlled by PBAN (Mas et al., 2000). In summary, it is more likely that the PBAN-affecting pheromone biosynthetic step in *D. punctatus* is a step(s) in or prior fatty acid synthesis starting from acetate to palmitic acid.

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