Stimulation of pheromone biosynthesis in the moth Helicoverpa zea: Action of a brain hormone on pheromone glands involves Ca2+ and cAMP as second messengers

Russell A. Jurenka, Iowa State University
Emmanuelle Jacquin, Cornell University
Wendell L. Roelofs, Cornell University

Available at: https://works.bepress.com/russell_jurenka/4/
Stimulation of pheromone biosynthesis in the moth Helicoverpa zea: Action of a brain hormone on pheromone glands involves Ca\(^{2+}\) and cAMP as second messengers

[pheromone-biosynthesis-activating neuropeptide/(Z)-11-hexadecenal/Lepidoptera]

RUSSELL A. JURENKA, EMANUELLE JACQUIN*, AND WENDELL L. ROELOFS†

Department of Entomology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456

Contributed by Wendell L. Roelofs, July 3, 1991

ABSTRACT Isolated abdomen and pheromone gland bioassays were utilized to determine the physiological action of the pheromone-biosynthesis-activating neuropeptide (PBAN) in the corn earworm moth Helicoverpa (= Heliothis) zea. An isolated pheromone gland bioassay showed that synthetic PBAN was active at 0.02 pmol, with maximal activity occurring at 0.5 pmol and 60 min of incubation. Second-messenger studies demonstrated that extracellular Ca\(^{2+}\) is necessary for PBAN activity on isolated pheromone glands. The Ca\(^{2+}\) ionophore A23187 stimulated pheromone biosynthesis alone, whereas the Ca\(^{2+}\) channel blockers La\(^{3+}\) and Mn\(^{2+}\) inhibited PBAN activity. However, the organic Ca\(^{2+}\) channel blockers verapamil and nifedipine did not inhibit PBAN activity. Both forskolin and two cAMP analogs stimulated pheromone biosynthesis in the absence of extracellular Ca\(^{2+}\), indicating that Ca\(^{2+}\) may activate an adenylate cyclase. The biogenic amine octopamine did not elicit pheromone production in isolated gland or abdomen bioassays or when injected into intact female moths. Removal of the ventral nerve chord, including the terminal abdominal ganglia in isolated abdomens, did not affect PBAN stimulation of pheromone production. Similar levels of stimulation were found when isolated abdomens were treated with PBAN in scotophase or photophase.

Sex pheromone titers in most moths, including the corn earworm moth Helicoverpa (= Heliothis) zea, fluctuate diurnally with little or no pheromone present during the day and with peak pheromone titers occurring during midscotophase (1). This fluctuation is caused by the release and/or degradation of existing pheromone and the biosynthesis of new pheromone at precise times of the photoperiod. The biosynthesis of pheromone in H. zea is regulated by a peptide produced in the subesophageal ganglion (SEG) portion of the brain complex of female moths (2). This peptide, termed pheromone-biosynthesis-activating neuropeptide (PBAN), has been isolated and sequenced from brains of H. zea (3). Two other pheromotropic peptides, each with about 80% sequence identity with H. zea PBAN, have been isolated from the brains of the silkworm, Bombyx mori (4, 5). These peptides have been synthesized and the synthetic peptides retain biological activity, with cross-reactivity occurring in other species of moths (3, 4, 6, 7). These reports and others indicate that a variety of moths utilize PBAN or PBAN-like peptides in controlling pheromone biosynthesis.

Although PBAN or PBAN-like activity has been identified in several different moth species, the physiological mode of action of PBAN has not been determined conclusively for any one species. In fact, there are two apparently conflicting hypotheses on how PBAN activates pheromone biosynthesis, and both are based on studies utilizing Heliothis moths as experimental animals. One hypothesis suggests that PBAN is synthesized in the SEG, transported to the corpora cardiaca, and then released into the hemolymph, where it can act on the pheromone gland to induce pheromone biosynthesis (8). The second hypothesis suggests that PBAN is transported from the SEG through the ventral nerve chord (VNC) to the terminal abdominal ganglia (TAG), and then another neurosecretory substance acts on the pheromone gland to induce pheromone production (9). A recent study reported that the pheromone gland is innervated and that the biogenic amine octopamine stimulates pheromone production, suggesting neural regulation of pheromone biosynthesis (10). However, in another report removal of the TAG in H. zea did not diminish PBAN stimulation of pheromone production, indicating that an intact VNC is not necessary for PBAN activity (11). Studies conducted with two other moths, the Asian cornborer, Ostrinia furnacalis (12), and the redbanded leafroller moth, Argyrotaenia velutinana (6), also indicate that an intact VNC or TAG is not necessary for PBAN activation of pheromone production. An intact VNC is apparently necessary for pheromone production in the gypsy moth, Lymantria dispar (13). However, there is no evidence to suggest PBAN control of pheromone production in female gypsy moths (R. E. Charlton and W. L. R., unpublished results), and another mechanism is probably acting to control pheromone biosynthesis. Other mechanisms implicated in the control of pheromone biosynthesis in other moths include juvenile hormone control of PBAN release in a migratory moth, Psuedaletia unipuncta (14), and the apparent lack of PBAN control in the cabbage looper, Trichoplusia ni (15). In the latter case there is evidence for ecdysone regulation of gland competency during the pupal stage (16).

Although the exact site of PBAN action has not been conclusively determined for any one species, recent reports indicate that PBAN stimulates \(^{14}C\)lacetate incorporation into pheromone by isolated pheromone glands of Helicoverpa armigera and H. zea (17–20). This activity is apparently mediated by the second messenger cAMP (21). Recently it was demonstrated that pheromone production could be stimulated in isolated pheromone glands of B. mori (22). In this study we demonstrate that PBAN can act directly on isolated glands of H. zea and that the second messengers Ca\(^{2+}\) and cAMP are involved in PBAN signal transduction.

Abbreviations: PBAN, pheromone-biosynthesis-activating neuropeptide; SEG, subesophageal ganglia; TAG, terminal abdominal ganglion; VNC, ventral nerve chord; Z11-16:Ald, (Z)-11-hexadecenal; 8Br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate; 8Br-cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate; 8BZIn-cAMP, 8-benzylaminoadenosine 3',5'-cyclic monophosphate.

*Present address: Institut National de la Recherche Agronomique, Laboratoire des Médiateurs Chimiques, Domaine de Brouessy, F-78114 Magny-les-Hameaux, France.

To whom reprint requests should be addressed.
MATERIALS AND METHODS

Materials. Synthetic PBAN was purchased from Peninsula Laboratories or was kindly supplied by A. Raina (U.S. Department of Agriculture, Beltsville, MD). The sodium salt of 8-bromoadenosine 3',5'-cyclic monophosphate (8Br-cAMP), 8-benzylaminoadenosine 3',5'-cyclic monophosphate (8BzIN-cAMP), the sodium salt of 8-bromoguanosine 3',5'-cyclic monophosphate (8Br-cGMP), LaCl3, MnCl2, (±)-verapamil-HCl, nifedipine, the insect's photophase, and forskolin, EGTA, ionophore nifedipine, the insect's photophase, otherwise indicated (20).

The VNC study, unless indicated otherwise, was performed during the insect's photophase, approximately 2–3 hr before lights off.

Isolated Abdomen and Gland Incubations. Isolated abdomens were prepared by cutting the abdomen away from the rest of the body at the fourth segment and placing the cut end of the exoskeleton into a drop of saline. The VNC was removed from these abdomens by making two incisions on either side of the ventral midline, exposing the VNC and TAG. The VNC and TAG were then separated from the ventral exoskeleton and placed in an 80-mm Petri dish with the cut end in contact with a 10-μl drop of saline containing PBAN. Sham-operated abdomens were prepared in the same way except the VNC was left intact. In some experiments the abdomens were excised between the sixth and seventh abdominal segments and the abdominal tip was incubated on a 10-μl drop of saline. These experiments were performed during the insect's photophase, approximately 2–3 hr before lights off.

Isolated glands were prepared as described previously (20). One gland per 5 μl of saline was utilized throughout this study.

All incubations were performed for 1 hr at room temperature unless indicated otherwise.

Analytical Methods. After the indicated incubation times, glands were removed and extracted for 15 min in 40 μl of hexane containing 40 ng of the internal standard (Z)-11-tetradecenal. The hexane extracts were analyzed directly by gas chromatography on a Carbowax 30-m capillary column (i.d. = 0.25 mm; Altech Associates) and a flame ionization detector. Pheromone [(Z)-11-hexadecenal (Z11-16:Ald)] was quantified by using synthetic standards and determining peak areas relative to the internal standard. Amounts were determined by comparing peak areas to the internal standard. Pheromone production was measured by the amount of Z11-16:Ald produced by an isolated gland. When incorporation of [1-14C]acetate into pheromone was measured, glands were extracted in hexane and the aldehydes were separated from other lipids by thin-layer chromatography and the amount of incorporation was determined by scintillation spectroscopy (6).

Statistics. In all experiments individual glands were analyzed and the differences among means were compared by analysis of variance followed by Fisher's least significant difference multiple range test. The data were transformed (y = log(1 + x)) when variances were unequal.

RESULTS

VNC Removal. Isolated abdomens exposed to PBAN during the photophase were stimulated to produce pheromone (Fig. 1). About 30 ng of pheromone was produced in a 1-hr incubation with 5 pmol of PBAN. Abdomens from which the VNC and TAG had been removed produced about 20 ng of pheromone when incubated with 5 pmol of PBAN. This was not significantly different from sham-operated abdomens treated with PBAN. Control abdomens incubated for the same period of time without PBAN produced about 2 ng of pheromone.

In another experiment abdomens were cut between the sixth and seventh segments and the excised abdomens, which lack the TAG, were utilized (Fig. 1B). These isolated abdomens were also stimulated by PBAN to produce pheromone as measured by the incorporation of [1-14C]acetate. Similar levels of incorporation were found when abdomens were removed from females and incubated during the photophase or removed from females just prior to the onset of scotophase and incubated for 1 hr in the dark (Fig. 1B).

Dose–Effect and Time–Response Curves. PBAN stimulated pheromone production in isolated glands in a dose-dependent manner (Fig. 2A). Maximum response was observed at about 0.5 pmol (0.1 μM) in this assay. No activity was observed at 5 fmol. Pheromone production was linear with increasing time of incubation up to 60 min (Fig. 2B).

Role of Ca2+ in PBAN Activity. Isolated glands were incubated in saline containing PBAN and with or without Ca2+. As shown in Fig. 3, PBAN was active only when glands were incubated in saline containing Ca2+, with 3 mM Ca2+ giving a maximal response. The Ca2+ ionophore A23187 was also effective in stimulating pheromone production as shown in Fig. 4. However, when glands were incubated in saline containing Ca2+ and the Ca2+ channel blocker La3+, PBAN was no longer active (Fig. 4). In separate experiments lower doses of La3+ did not inhibit PBAN stimulation of pheromone production (1 pmol of PBAN plus 0, 0.1, or 0.5 mM La3+ gave 110 ± 20, 108 ± 17, or 99 ± 15 ng of Z11-16:Ald per gland, respectively). However, 2 mM La3+ was inhibitory (18 ± 4 ng of Z11-16:Ald per gland). Another inorganic Ca2+ blocker, Mn2+, also inhibited PBAN stimulation (1 pmol of PBAN plus 0, 2, or 5 mM Mn2+ gave 140 ± 17, 70 ± 10, or 52 ± 6 ng of Z11-16:Ald per gland, respectively). Pretreatment for 20 min with the organic Ca2+ chan-

![Fig. 1. Pheromone production during incubation of isolated abdomens of female H. zea with PBAN. (A) Abdomens (segments 4–9) were left unoperated (unop), or the VNC including the TAG were removed (minus VNC), or the abdomens were operated on but the VNC were left intact (sham). These abdomens were treated with 5 pmol of PBAN. Control abdomens (con) were left intact and were not treated with PBAN (n = 5). (B) Incorporation of [1-14C]acetate into pheromone was measured in isolated abdomens (segments 7–9), which lack the TAG) that were incubated with or without 2 pmol of PBAN during the photophase or at the onset of scotophase. Bars represent the mean ± SEM with the number of replicates indicated at the top of each bar in B; different letters indicate groups statistically different at P < 0.05.](image-url)
beside (A) or pheromone plus pmol of gland 8BzlN-cAMP. The cAMP of stimulation of Ca2+ blockers verapamil and nifedipine did not inhibit PBAN stimulation of pheromone production in isolated glands (1 pmol of PBAN plus 0, 0.1, or 2 mM verapamil gave 110 ± 20, 178 ± 19, or 85 ± 11 ng of Z11-16:Ald per gland; 1 pmol of PBAN plus 0, 10, or 100 μM nifedipine gave 128 ± 20, 94 ± 16, or 87 ± 6 ng of Z11-16:Ald per gland, respectively). All of the channel blocker experiments were conducted with saline containing 3 mM Ca2+.

Effect of cAMP and Forskolin. The cAMP analogues 8Br-cAMP and BzIzN-cAMP stimulated pheromone production in isolated gland incubations, whereas the cGMP analogue 8Br-cGMP had no effect on pheromone production (Fig. 5). The 8BzIzN-cAMP analogue was active in a dose-dependent manner, with 5 mM producing a maximal response. However, similar levels of stimulation were observed when this analogue was tested at 0.5 mM with various concentrations of Ca2+ (Fig. 5 Upper). 8Br-cAMP was also active when incubated with isolated glands in Ca2+-free saline. Although the total amount of pheromone produced was not significantly different when 8Br-cAMP was incubated with glands in saline with or without Ca2+, a lower amount was produced in incubations without Ca2+. A similar result was also observed when the adenylate cyclase inducer forskolin was incubated with glands in saline with or without Ca2+ (Fig. 5).

Effect of Octopamine. As shown in Fig. 6, octopamine at the indicated concentrations did not stimulate pheromone production when incubated with isolated glands. However, in parallel experiments in which PBAN was incubated with isolated glands, pheromone production was increased. Likewise, PBAN, but not octopamine, stimulated pheromone production when injected into intact females during the photophase (Fig. 6). In another experiment, incubation of isolated abdomens (segments 7–9), taken 1–2 hr before lights off, with 5 pmol (1 μM) of octopamine did not produce a significant change in incorporation of radiolabeled acetate into pheromone (21 ± 1 cpm per gland, n = 8) compared with control values (40 ± 13 cpm per gland, n = 8). However, isolated abdomens treated with 2 pmol of PBAN incorporated larger amounts of radiolabeled acetate into pheromone (1409 ± 201 cpm per gland, n = 8) than controls. The octopamine utilized here was also tested in male cabbage loopers by injecting 3 μg and observing their response behaviorally to a pheromone source as described by Linn and Roelofs (23). This test was positive, indicating that the octopamine was biologically active. In all of the octopamine experiments the following saline was utilized: KCl, 3 mM; NaCl, 150 mM; CaCl2, 3 mM; MgCl2, 20 mM; sucrose, 25 mM; and Pipes buffer, 10 mM brought to pH 6.9 with KOH. This saline is
FIG. 5. Pheromone production during incubation of isolated glands of *H. zea* with the cyclic nucleotide analogues 8Br2N-cAMP (Upper) or 8Br-cAMP, 8Br-cGMP, and the adenylate cyclase inducer forskolin (Lower). (Upper) □. Amount of pheromone produced at various concentrations of 8Br2N-cAMP and 3 mM Ca\(^{2+}\) (n = 0–12); △, amount of pheromone produced at 0.3 mM 8Br2N-cAMP and various concentrations of Ca\(^{2+}\) (n = 8). (Lower) CNMPs were tested at 2 mM. 8Br-cAMP and forskolin were tested in saline with or without Ca\(^{2+}\). Saline without Ca\(^{2+}\) contained 1 mM EGTA. Control glands were incubated in saline with Ca\(^{2+}\). Bars represent the mean ± SEM, with the number of replicates indicated at the top of each bar; within the four bars on the left or the five on the right, different letters indicate statistically different (P < 0.05) groups.

similar to one employed by Christensen et al. (10), who reported that Mg\(^{2+}\) treatment (20 mM) of the TAG blocked the electrical stimulation response from the TAG to the pheromone gland, apparently by disrupting Ca\(^{2+}\)-mediated synaptic transmission. They also reported that octopamine was stimulatory when injected into females and that PBAN did not stimulate isolated pheromone glands (10).

**DISCUSSION**

Our results, along with those of others (17–19), indicate that PBAN acts directly on pheromone glands of *Helicoverpa* and *Heliothis* moths to stimulate pheromone biosynthesis and does not involve elements of the nervous system. In the present study isolated abdomens and glands that did not contain the TAG were stimulated by synthetic PBAN to produce pheromone. Isolated glands (abdominal segments 8 and 9) of *H. zea* and *H. armigera* were also stimulated to produce pheromone when incubated with a brain homogenate or with synthetic PBAN (17–19). However, Teal et al. (9) reported that isolated pheromone glands of *H. zea* were not stimulated by PBAN and presented evidence that intact nerves leading from the TAG are required for PBAN activity. They suggested that a second neurosecretory substance released from the TAG actually stimulated the pheromone gland. A recent report indicated that the biogenic amine octopamine stimulated pheromone production when injected into females of *Heliothis virescens* and *H. zea* (10). However,

the data presented here support the previous findings by us and others that the TAG is not necessary for pheromone production in several different moths (6, 11, 12, 17–20, 22). Additionally, using isolated glands or abdomens or intact females, we could not stimulate pheromone production with octopamine. PBAN, however, causes a high and consistent increase in pheromone biosynthesis in isolated glands of *H. zea* (this work and refs. 18 and 20) and *H. armigera* (19).

Production of sex pheromone in *H. zea* cycles diurnally, with the highest titers occurring during midscotophase and almost no pheromone present during photophase. However, PBAN stimulation during the light period was demonstrated in vitro by using isolated glands of *H. zea* (this work and refs. 19 and 20) and *H. armigera* (17, 18). Nevertheless, two other reports indicate that a dark period is required for PBAN activity. In one report increased radiolabeled acetate incorporation into pheromone was observed when isolated glands of *H. armigera* were incubated with PBAN in the dark (17). The other report indicated that both PBAN and octopamine were stimulatory when incubated during the first hour of scotophase with isolated abdomens of *H. virescens* lacking the TAG but were not stimulatory when incubated during photophase (10). However, we did not observe any differences in the stimulation of radiolabeled acetate incorporation into pheromone when isolated abdomens of *H. zea* were incubated with PBAN during photophase or at the onset of scotophase.

In a previous study (20) we showed that PBAN stimulates the incorporation of radiolabeled acetate into pheromone. The incorporation of radiolabeled acetate into pheromone also increased in isolated glands of *H. armigera* incubated with synthetic PBAN (19). In the report by Rafaeli et al. (19) about 5 pmol (0.05 μM) of synthetic PBAN gave maximal stimulation. This amount is similar to that reported here, where 0.5 pmol (0.1 μM) gave a maximal response. A difference noted between the present study and the one with *H. armigera* is that a 3-h longer incubation time was required to reach maximal pheromone production in the latter study. The longer incubation time needed in the study with *H. armigera* may reflect differences in bioassay technique. The isolated glands of *H. armigera* were abdominal tips that were essentially conical and exposed to incubation media only through one end. Isolated glands in the present study were made such that the glands cells were directly exposed to the saline, allowing for maximal contact with test substances.

Rafaeli and colleagues (19, 21) have shown that cAMP analogues stimulate pheromone biosynthesis in *H. armigera* and that PBAN stimulates the production of cAMP in the
However, isolated prothoracic glands are stimulated by the prothoracicotropic hormone, a peptide, to biosynthesize and secrete ecdysone without nervous stimulation. The prothoracicotropic hormone probably acts on a membrane receptor found on cells in the prothoracic gland (28). Evidence presented in the present paper suggests that PBAN is acting directly on prothoracic gland cells through a receptor-activated Ca\(^{2+}\) channel. However, further research is needed to identify the PBAN receptors.

We thank Ashok Raina for supplying the synthetic PBAN, Kathy Poole and Marlene Campbell for rearing the insects, and Charlie Linn for testing the biological activity of the octopamine used in this study. This study was supported by the National Science Foundation (Grant DCD-8711955).