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FINAL REPORT

PROJECT NO. IS-1645-89

**Mechanisms Involved in the Neurohormonal
Regulation of Sex Pheromone Production in
Lepidoptera**

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ROYAL
CANADIAN
MOUNTED
POLICE

1995

Date: 21st August, 1995

BARD Project Number: IS 1645-89

Project Title:

MECHANISMS INVOLVED IN THE NEUROHORMONAL
REGULATION OF SEX PHEROMONE PRODUCTION IN
LEPIDOPTERA.

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Project Start Date: September, 1991

Type of Report:

1st Annual

2nd Annual

Final ✓

Signature:

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Signature:

Authorizing Official, Affiliated Institution

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Abstract

By our studies, conducted on pheromone gland cultures *in vitro*, we have delineated the pheromone gland to be confined to the intersegmental membrane between the 8th and 9th abdominal segments. We concluded that the control mechanism, which exists at specific periods and is induced by PBAN (pheromone biosynthesis activating neuropeptide), is not at the level of the target tissue since pheromone gland cultures from both virgin and mated females and from all ages and photoperiods are fully competent to PBAN stimulation *in vitro*. Studies using synthetic PBAN analogs showed that the essential site for biological activity resides in the C-terminal portion of the peptide. PBAN was found to induce a complex multi-signal transduction system involving cAMP, calcium and phosphatidyl inositol breakdown. We have evidence of "cross-talk" between these transducing systems. An antiserum raised against synthetic PBAN has been used in immunocytochemistry to localize PBAN immunoreactive cells. We also developed a sensitive and specific RIA and ELISA to determine levels of PBAN immunoreactivity in various tissues taken from females of various ages and photoperiods. Females in photophase do not produce pheromone unless injected with PBAN. We found that starved photophase females which were fed PBAN or analogs in a sucrose solution would produce pheromone as if they were injected with PBAN. Following the course of radiolabelled PBAN analogs we were able to show that these peptides can traverse the wall of the alimentary canal and reach the target tissue via the hemolymph. From studies on other moth species (*Lymantria dispar*, *Pectinophora gossypiella* and *Plodia interpunctella*) the involvement of PBAN or a PBAN-like endogenous factor was clearly shown, however in some moth species (gypsy moth and pink bollworm moth) the intervention of a nervous element was also evident since an enhanced pheromonotropic response to PBAN was achieved after severance of the ventral nerve cord. Biogenic amines such as octopamine and tyramine were observed to inhibit the pheromonotropic action due to PBAN both in pheromone gland incubations as well as in intact females during the scotophase and the photophase. This inhibitory effect was also evident on intracellular cAMP production. Mating and sex pheromone production by females was shown to be inhibited by the transfer of a peptide (PSP) from the male accessory glands to females during copulation. Pheromone production in these females could be restored by the injection of PBAN. The action of PSP was also found to be dependent on an intact ventral nerve cord.

Objectives of the original research proposal

In the present proposal our main goal was to evaluate the hormone status of PBAN and define its physiological role in regulating pheromone biosynthesis. To achieve this goal we planned to: (1) determine the spatial and temporal distribution of PBAN; (2) identify, immunocytochemically, the neurosecretory cells responsible for the production and storage of PBAN (3) determine the release sites of PBAN and investigate the factors influencing its release and (4) elucidate the receptor site for PBAN.

During the progress of the above objectives we realized from current research reports that although PBAN was shown to stimulate pheromone production in some species, especially those belonging to the Noctuidae, in other species additional or different factors were reported to play a role in pheromone production. This led to much speculative suggestions of the mode of action of pheromone regulation in moths. Since very few species have been studied to date, and since in each study various techniques were utilized, much discrepancy has accumulated. We therefore also initiated a series of studies using various other species of moths belonging to families other than the Noctuidae and included a 5th objective to study the occurrence of PBAN in other species.

Relevant data, methodology, results and discussion

Target tissue (Elucidation of receptor site =Objective 4)

Through the development of a sensitive *in vitro* bioassay, we were able to present evidence, for the first time, that isolated pheromone glands of *Helicoverpa zea* and *H. armigera* are target tissues for PBAN (Rafaeli *et al.*, 1993; Rafaeli, 1994; Rafaeli & Gileadi, 1995a). Pheromonotropic activity was obtained in the absence of the terminal abdominal ganglion (TAG) connectives in *H. zea* and the direct neurohormonal control of pheromone biosynthesis by PBAN was demonstrated in both *Helicoverpa* species (*zea* and *armigera*) using pheromone gland cultures *in vitro*. Pheromone gland activation involved the *de novo* production of the main pheromone component (Z)-11-hexadecenal as revealed by radio-TLC, HPLC and GC. Activation was found to be a specific response attributed to pheromone gland cultures alone. Moreover, in a recent investigation, we have delineated the pheromonotropic activity to be confined only to the intersegmental tissue which is situated between the 8th and 9th abdominal segments of the ovipositor tip (Rafaeli & Gileadi, 1995a). No pheromonotropic activity (represented by pheromone biosynthesis and intracellular cAMP formation) was obtained in incubations of the 8th segment alone when challenged with PBAN (Rafaeli & Gileadi, 1995a). Specificity of pheromonotropic activation was demonstrated to be limited to nervous tissue extracts. Since pheromone glands were fully competent to

produce pheromone *in vitro* during all photoperiods and ages as well as after mating we concluded from these studies that the control mechanism of photoperiodically-controlled pheromone production in these species resides in the control of PBAN processing and release from the nervous system and it is not at the level of the target tissue.

Structure activity studies (Elucidation of receptor site =Objective 4)

Studies using synthetic PBAN analogs showed that the essential site for biological activity resides in the C-terminal pentapeptide sequence (FXPRLamide, Xaa=Ser or Thr) (Raina & Kempe, 1992). The N-terminal, while not active by itself, is essential for full potency of pheromonotropic activity. Dose response studies using PBAN and a hexapeptide C-terminal fragment of PBAN suggested that PBAN might be inducing a two mechanism response, one occurring at low PBAN concentrations and another at higher PBAN concentrations (Rafaeli, 1994). Further evidence indicating a multi-site/receptor system was obtained with the observation that other second messengers are involved in transducing the PBAN response (see below).

Second messengers (Elucidation of receptor site =Objective 4)

Most neuropeptides interact with surface receptors to induce a cascade of intracellular second messengers which amplify the initial peptide-receptor signal. These may be small organic molecules such as: cAMP, cGMP; ions, such as calcium and hydrogen; or inositol phosphates. Our studies concerning the cellular and physiological response of the pheromone gland *in vitro* revealed the direct involvement of several second messengers (cAMP, Ca^{2+} and phosphoinositol breakdown)(Rafaeli, 1994; Rafaeli & Soroker, 1994; Soroker & Rafaeli, 1995).

The pheromonotropic response to PBAN was observed to induce increases in intracellular cAMP levels in *H. armigera*. Further evidence of the involvement of cAMP as second messenger was obtained with pharmacological agents such as forskolin (an adenylate cyclase activator), isobutyl methyl xanthine (IBMX, a phosphodiesterase inhibitor) and cAMP analogs such as 8-bromo-cAMP, dibutyryl cAMP and SpcAMPS which were able to mimic the pheromonotropic response. Calcium was found to be essential for pheromonotropic activity and was implicated in the mediation of the pheromonotropic response in *H. armigera*. Pheromone production was stimulated by calcium ionophores in gland preparations *in vitro*. These observations indicated that both second messenger systems were involved in mediating the pheromonotropic action of PBAN. Dose response studies have indicated that there may be more than one receptor or receptor sites for PBAN since saturation of the pheromonotropic response (pheromone titres) occurs at lower PBAN levels than those required for saturation of the cellular response (intracellular cAMP levels). This may indicate that there may be different affinity sites for the PBAN receptor.

The involvement of a third messenger system was implicated by the stimulation of pheromone biosynthesis by the phorbol ester, PMA and the diacylglycerol analog OAG both of which stimulate C-kinase dependent protein phosphorylation. The action of PMA was also found to be calcium dependent. The interaction of the different messenger systems was further studied using various inhibitors of protein kinases, and calcium-calmodulin. Inhibition of both pheromone biosynthesis and cAMP formation was observed by the calcium-calmodulin inhibitor, W12 although no inhibition was observed with staurosporine, a protein kinase C inhibitor. Omission of calcium from the incubation medium completely abolished the increase in intracellular cAMP levels due to PBAN stimulation. However, the stimulation as a result of the cAMP analog Sp-cAMPS was not dependent on the presence of calcium in the incubation medium thus indicating that pheromone biosynthesis, beyond the level of cAMP formation, does not require extracellular calcium. These results suggest that calcium, entering the cell as a result of the interaction of PBAN with its receptor, activates adenylate cyclase. Indeed, we found that the calcium ionophores (ionomycin and thapsigargin) significantly stimulated the production of intracellular cAMP in pheromone glands of *H. armigera* (Soroker & Rafaeli 1995; Rafaeli, unpublished).

It therefore appears that the activation of pheromone production can operate via the stimulation of adenylate cyclase and/or the phosphatidylinositol pathway. In both systems calcium is crucial for activation. However, whether the activation of these systems is due to the interaction of several receptors or receptor sites and the relationship between these systems is still unclear. Clarification of this must await isolation and identification of the PBAN receptor/s.

Immunocytochemical identification of PBAN producing cells in SOG (=Objective 2)

The question arose as to how PBAN reaches its target tissue? Is it transported in the hemolymph or down the ventral nerve cord? Using the antiserum to PBAN we investigated the distribution of immunoreactive peptide in the brain-suboesophageal ganglion complex (Brain-SOG), its associated neurohemal structures and the ventral nerve cord. Immunocytochemical methods revealed three clusters of cells along the ventral midline in the suboesophageal ganglion. Of these cells, members of the labial cluster project axons to the retrocerebral complex (corpora cardiaca and cephalic aorta). In addition, two pairs of axons from the maxillary cluster project into the ventral nerve cord (Kingan *et al.*, 1992; Blackburn *et al.*, 1992). From these observations it is conceivable that PBAN travels both down the ventral nerve cord as well as into the corpora cardiaca.

Distribution of PBAN immunoreactivity and identification of release sites
 (=Objective 1 & 3)

A sensitive and specific radioimmunoassay (Rafaeli *et al.*, 1991; 1993) as well as an ELISA (Kingan *et al.*, 1992) were developed using [3H]-PBAN (for radioimmunoassay) and an antiserum to PBAN. The spatial and temporal distribution of PBAN-immunoreactivity throughout the nervous system was studied using this RIA (Rafaeli *et al.*, 1991; 1993) and ELISA (Kingan *et al.*, 1992). PBAN-immunoreactivity in brain complexes was found throughout the photoperiod and in all ages. Observations of levels of immunoreactive PBAN obtained using the RIA showed that in brain-suboesophageal extracts relatively high levels of PBAN-immunoreactivity were maintained throughout the photoperiod. Levels rose significantly during the beginning to the middle of photophase and declined towards the end of photophase remaining stable throughout the scotophase. In the corpora cardiaca, on the other hand, lower levels were observed during the photophase rising during the scotophase. These studies supported the hypothesis that PBAN is continuously synthesized in the suboesophageal ganglion and accumulates during the photophase. It can be conceived that at this period gating of PBAN passage to the corpora cardiaca is closed. Towards the end of photophase the gate is opened and this allows the release of PBAN into the neurohemal gland. These findings suggest that the corpora cardiaca release the hormone into the hemolymph. Other nerve tissues were also tested for PBAN-immunoreactivity and, although levels were found to be much lower than those found in brain-suboesophageal complexes, in the TAG higher levels were observed at the beginning of the photophase when compared to the scotophase. These findings suggested an accumulation of immunoreactive-PBAN in the TAG during the photophase and a depletion during the peak hours of pheromone production and therefore support the alternate hypothesis which suggested that PBAN travels down the ventral nerve cord. It should however be noted that the antiserum to PBAN used in these studies recognizes the C-terminal pentapeptide sequence which has subsequently been found to represent a common sequence for many peptides found in the nervous system.

We demonstrated that PBAN and its smaller biologically active analogs when fed to *H. zea* females during photophase can pass the gut wall and induce pheromone production. Intact PBAN and radioactive analogs were found intact in the hemolymph of such fed females (Raina *et al.*, 1994).

Several natural corn silk volatiles, including the plant hormone ethylene, induced pheromone production in *H. zea* females. Exposure to the host plant caused the loss of immunoreactive-PBAN from the corpora cardiaca, suggesting that host plant signals could induce the release of PBAN from the neurohemal organ, leading to the production of the pheromone (Raina *et al.*, 1992).

Occurrence and action in various moth species (=Objective 5)

PBAN was characterised from the gypsy moth *Lymantria dispar* and was shown to have an 81.8% sequence homology to Hez-PBAN (Masler *et al.*, 1994). In physiological studies concerning *Lymantria dispar* (Lymantriidae), we observed that although ligation between the head and thorax caused termination of pheromone production, injections of PBAN were only stimulatory when the ventral nerve cord was cut just before injection (Thyagaraja *et al.*, 1994). We observed a similar phenomenon in the pink bollworm moth, *Pectinophora gossypiella* (Gelechiidae) (Rafaeli & Klein, 1994). This indicated that, in these species, unlike our findings on *Helicoverpa zea* and *H. armigera*, a possible neural element is involved in the control of pheromone production. On the other hand *Plodia interpunctella* (Pyraliidae), although belonging to a family other than Noctuidae, responded similarly to PBAN (Rafaeli & Gileadi, 1995b).

Suppression after mating (Factors influencing release of PBAN=Objective 3)

Mating and sex pheromone production by females was shown to be inhibited by mating in *Plodia interpunctella* (Rafaeli & Gileadi, 1995b). In *Helicoverpa zea* this inhibition was attributed to the transfer of a peptide from the male accessory glands to females during copulation (Kingan *et al.*, 1995). The peptide evoked the depletion of pheromone when injected into virgin females. This peptide was characterised as a 57 amino residue with one disulfide bridge and a blocked N-terminus with pyroglutamate and an amidated C-terminal. Our studies indicate that this peptide inhibits the release of PBAN and its target tissue is most probably neural since females whose abdominal nerve cords have been severed are not depleted of pheromone after mating.

Aminergic modulation (Receptor site for PBAN = Objective 4)

It has been proposed by other workers that the biogenic amine, octopamine, is involved in the regulation of sex pheromone production in Lepidoptera. This proposal has been the outcome of observations by these workers that PBAN activity in *H. zea* and *H. virescens* requires the presence of an intact ventral nerve cord (VNC). These workers explained this observation to indicate that PBAN triggers the terminal abdominal ganglion (TAG) to release octopamine into a network of nerves innervating the pheromone gland and that it is octopamine (and not PBAN) which is responsible for the direct stimulation of pheromone biosynthesis.

However, research in our laboratories as well as other laboratories, using several species of moths, including *H. zea*, provide conflicting evidence to this theory where octopamine injections to intact females or addition to incubations of pheromone glands *in vitro* did not stimulate pheromone production (Rafaeli *et al.*, 1995). As mentioned above, using *H. armigera* pheromone gland incubations *in vitro* (Rafaeli *et al.*, 1991;

Rafaeli, 1994) we demonstrated that PBAN stimulated pheromone biosynthesis directly. In support of these findings, using several other moth species, pheromonotropic activity by PBAN was obtained in pheromone gland incubations and intact females devoid of the VNC (Raina *et al.*, 1991). We showed that prior depolarization of the tissue for 10-30 mins, using high levels of extracellular K^+ , which should effectively release any neurotransmitter, failed to prevent the normal pheromonotropic response obtained with PBAN (Rafaeli *et al.*, 1995).

On the other hand, we suggested a modulatory role for octopamine by our findings that the PBAN-induced pheromonotropic activity of intersegmental membrane incubations, was significantly inhibited in a dose-dependent manner by the presence of biogenic amines such as octopamine (minimum concentration of $10^{-5}M$) (Rafaeli & Gileadi, 1995a), tyramine (minimum concentration of $10^{-7}M$) and clonidine (minimum concentration of $10^{-9}M$). This inhibitory effect was found to be dependent on the PBAN concentration in the incubation medium and reversed using aminergic antagonists such as phentolamine and yohimbine (Rafaeli & Gileadi, 1995a).

Since pheromonotropic activity is mediated by cAMP the effect of octopamine on cAMP levels was also studied. The significant pheromonostatic action of octopamine was also evident by the inhibition of the production of intracellular cAMP (Rafaeli & Gileadi, 1995a). In addition, the pheromonostatic action of octopamine was confirmed by experiments *in vivo* in both intact photophase (Rafaeli & Gileadi, 1995a) and scotophase females (Rafaeli, in preparation).

Description of Cooperation

The investigators exchanged trips to the US and Israel respectively. The US lab developed the antiserum to PBAN which was used in the Israeli lab. for immunoreactivity studies. This enabled comparisons using the same antiserum. Synthetic PBAN and analogs used in the Israeli lab. were also developed in the US lab. Studies on oral feeding of PBAN were conducted during a trip to the US lab. by the principal investigator. Several additional meetings for exchange of information were arranged between the PI and CI during scientific meetings in the US and Europe.

Evaluation of the research achievements

Our studies have successfully evaluated the hormone status of PBAN and defined its physiological role in regulating pheromone production. We have determined the spatial and temporal distribution of PBAN and shown the exact site of synthesis thereby completing objectives (1) and (2). Release sites were determined and we found that mating suppresses release of PBAN thereby completing objective (3). In addition the receptor site for PBAN was elucidated, its receptor-effector responses were

characterized and we found that biogenic amines can interfere with this response. This concluded our 4th objective. We extended the original proposal to include some studies using various other moth species for comparison and by these studies we have discovered that most probably a nervous element, possibly inhibitory, is also involved in the feedback regulation of pheromone production.

Conclusions

Sex pheromones, as tools for the control of insect pests, have limited applications and are mainly used for monitoring insect infestations. Before strategies can be devised for using pheromones to control pest populations there is a need to broaden basic knowledge concerning the balanced regulation of the production of sex pheromones.

Our present study has advanced our understanding of the mode of action of PBAN. It has unequivocally determined the exact target tissue for PBAN and has shown that receptor-effector recognition occurs with the concomitant cascade of second messengers. It has shown that PBAN is produced in the brain-suboesophageal ganglion, stored in the neurohemal organs, the corpora cardiaca, and can reach the target tissue via the hemolymph. It has shown that receptor-effector recognition occurs with the concomitant cascade of second messengers. We have also a greater understanding of the mechanism by which suppression or interference of the receptor-effector cascade by biogenic amines leads to an effective inhibitory response in pheromone production.

A study of the nature of activation of the pheromone biosynthetic pathway by the protein kinases which are stimulated by PBAN will avail us of valuable knowledge concerning the receptor site. The research has also revealed to us a possible neural pheromonostatic intervention modulating pheromonotropic activity both during reproductively inactive periods as well as during mating. The availability of potential inhibitors as well as sensitive bioassays will greatly facilitate future progress in this research.

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