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Histochemical localization of the PBAN receptor in the pheromone gland of *Heliothis peltigera*

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Abstract

The presence of the pyrokinin (PK)/ Pheromone biosynthesis activating neuropeptide (PBAN) receptor in pheromone gland cells of *Heliothis peltigera* females was demonstrated, and its spatial distribution in the ovipositor was visualized with two photo-affinity biotinilated ligands: BpaPBAN1-33NH$_2$ and BpaArg^{27}-PBAN28-33NH$_2$. Light microscopy histological studies revealed that the gland is contained within the inter-segmental membrane (ISM) between the 8th and 9th abdominal segments. The gland was found to be composed of a single layer of columnar epithelial cells positioned under the inter-segmental cuticle. Similar epithelial cells were also found in the dorsal and ventral regions of the 9th abdominal segment. All regions containing the glandular cells bound both ligands, indicating presence of the PK/PBAN receptor. The patterns obtained with both ligands were similar, hinting at the possibility that either both ligands bind to the same receptor, or, that if there are two distinct receptors, their spatial distribution throughout the gland is very similar.
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1. Introduction

The sexual communication between sexes in Lepidopteran species is mediated mainly by sex pheromones, which are volatile compounds used by Lepidopteran insects to attract potential mates from a distance [20]. Sex pheromones play an important role in the elicitation of mating behavior in moths and are, therefore, crucial for successful mating and maintenance of reproductive isolation. Understanding the mechanisms that underlie sex pheromone production is, therefore, of major interest and importance.

Sex pheromones in Lepidopteran species are synthesized in a specialized gland, which is a modification of the inter-segmental membrane (ISM) located between the 8th and 9th abdominal segments. The pheromone-producing cells are epithelial cells, overlaid by a modified ISM cuticle, which, in most Lepidopterans is produced by the cells themselves. The pheromone is produced within the epithelial cells, transported through the cuticle *via* special porous cuticular spines, and is disseminated from the surface [33,41].

Sex pheromone biosynthesis in moths is affected by a variety of exogenous and endogenous factors (such as temperature, photoperiod, host plants, age, mating, as well as hormonal and neurohormonal factors). A major breakthrough in our understanding of the endogenous factors involved in this process occurred in 1984, when Raina and Klun [37] first reported that sex pheromone production in female *Helicoverpa* (then *Heliothis*) *zea* moths is controlled by a cerebral neuroendocrine factor, which they termed pheromone biosynthesis activating neuropeptide (PBAN). PBAN was found to control sex pheromone biosynthesis in many other moth species, and the peptide itself was found in many Lepidopteran species as well as in other insect orders [36]. PBAN was first isolated and characterized as a 33 amino acid C-terminally amidated neuropeptide by Raina et al. [38] in *H. zea*, and then its primary sequence was determined in numerous other moth species [17,36]. Determination of the primary amino acid sequence of PBAN revealed that its C-terminal pentapeptide sequence (FSPRLNH$_2$) is homologous with the C-terminal pentapeptide sequence of leucopyrokinin (LPK), the first pyrokinin (PK) peptide, which was isolated from the Maderae cockroach, *Leucophaea maderae* [28]. LPK, PBAN and other

pheromonotropic (e.g. pheromonostatin) and myotropic [29] peptides were all grouped into a family termed the PK/PBAN family, characterized by a structural homology at their C-terminus pentapeptide sequence (FXPRLNH$_2$; X = S, T, G, V) [17,36], which composes their active core [2–5,25,29,32,39,40].

The PK/PBAN family of peptides is a multifunctional family of peptides. In addition to their ability to stimulate sex pheromone biosynthesis in moths, they mediate key functions associated with feeding (gut muscle contractions) [28,42], development (pupariation and diapause) [21,30,31] and defense (melanin biosynthesis) [3,26] in a variety of insects (moths, cockroaches, locusts and flies) (for review see [17,36]). Currently, ca. 15 peptides have been identified (including pyrokinins, myotropins, PBAN, melanization and reddish coloration hormone (MRCH), diapause hormone and pheromonostatin) [17,36]. Studies performed in several laboratories including ours have shown that sex pheromone production (as well as all of the other above functions) can be stimulated by more than one peptide and that the peptides do not exhibit species specificity [17].

Although it is generally agreed that pheromone production in many Lepidopteran species involves the participation of PBAN and possibly other members of the family, many questions still remain unanswered. Currently, very little is known about the mechanism of action of PBAN and other members of this family, and much remains to be determined about the structural, chemical and cellular bases of their activity. Even the nature of the peptide(s) that actually elicit pheromonotropic activity in vivo (full-length PBAN or one of the other pheromonotropic peptides that share sequence homology with it) is not known yet.

One reason for our lack of knowledge results from the fact that most studies on the role of the PK/PBAN peptides in pheromone biosynthesis are based on in vivo bioassays (e.g. [1,18,37]) in which peptides are injected into the hemolymph of insects under conditions in which sex pheromone production does not occur naturally (photophase or decapitated females as scotophase). Despite their great advantage in providing a good estimate of the bioactivity of the tested compounds such bioassays do not enable us to address the above issues, specifically those related to the target tissue on which PBAN acts and the downstream cellular events. Contradictory results that accumulated in the course of the years, regarding the principal site(s) of action of the neuropeptide (pheromone gland vs. other organ) and the possible involvement of additional factors in sex pheromone biosynthesis complicated the issue even further. For example, Teal et al. [47] demonstrated that the prime target of PBAN is the terminal abdominal ganglion (TAG), which in turn provides a signal to the gland to produce pheromone. Supportive of this theory were the findings of Christensen and co-workers [10,11], who demonstrated that sex pheromone production in *H. zea* and *H. virescens* is elicited by the biogenic amine octopamine in the absence of PBAN, in an age- and photoperiod-dependent manner.

These workers suggested that PBAN activates the TAG, and that this in turn secretes octopamine that activates the gland.

The bursa copulatrix has also been suggested as a potential target for PBAN. In a study performed by Jurenka et al. [23] in *Argyrothaenia velutinana*, it was demonstrated that abdomen cultures responded to a much greater extent than the pheromone gland to exogenously applied synthetic PBAN, and that the bursa copulatrix was essential for a full stimulatory response to PBAN. The study suggested that a pheromonotropic factor, other than PBAN, originating in the bursa copulatrix, is essential for pheromonotropic activity and that the role of PBAN is to stimulate the release of such a factor. The involvement of a bursal factor in the pheromonotropic activity has been reported in other moths as well [13]. Furthermore, experiments performed on *Trichoplusia ni*, *Agrotis segatum*, *A. velutinana* and on the pink bollworm *Pectinophora gossypiella* with pheromone glands in vitro failed to show pheromone biosynthesis in response to application of brain-subesophageal (SOG) extracts or synthetic PBAN [35,45,50]. All of the above studies hinted at the possibility that PBAN may act on a target other than the pheromone gland.

Other studies, though, clearly identified the pheromone gland as the prime target of PBAN. These studies demonstrated that in vitro gland cultures or preparations of ovipositor tips from a variety of insects could easily be stimulated to produce sex pheromone by application of brain extracts as well as of synthetic PBAN [15,34,43]. Anatomical evidence provided direct proof for the existence of PBAN release sites, presumably in the region of the pheromone gland [12,19,24], and recent studies demonstrated that viable pheromone gland cell clusters from the ISM could produce pheromone in response to a pheromonotropic peptide [16].

One way to get a better insight into the above issues and to resolve some of the above contradictions is by a direct demonstration of the presence of PBAN receptors on the pheromone gland cells. Receptors are central to the understanding of the biological function of any neuropeptide (especially in families where several peptides exhibit similar bio-activities) and central to prove a direct correlation between the activity of a given neuropeptide and its target.

Currently, very little is known about the PBAN receptor. No one has shown its cellular localization in the pheromone gland cells (or in any other tissue) and no information exists about its spatial distribution within the glandular area. It has also not been determined whether one or multiple receptors mediate sex pheromone biosynthesis in moths, and whether the same or different receptors mediate the various functions elicited by peptides of the PK/PBAN family in moths and other insects. In a previous study we used a radiolabeled ligand to develop a radio-receptor assay (RRA) that enabled us to partially characterize the PK/PBAN receptor of the pheromone gland of *Heliothis peltigera* females and to determine its expression under different conditions [6,7]. In the present study we have used two biotinilated photo-affinity (benzophenon substituted) PBAN ligands, a

full-length PBAN1-33NH$_2$ molecule and a shorter fragment derived from its C-terminus Arg^{27}-PBAN28-33NH$_2$, to demonstrate the presence of the PBAN receptor in pheromone gland cells of *H. peltigera* females and to determine its spatial distribution in the ovipositor.

2. Material and methods

2.1. Insects

H. peltigera moths were reared on an artificial diet as described previously [14]. Pupae were sexed and females and males were placed in separate rooms with a dark/light regime of 10 h:14 h, at 25 ± 2 °C and 60–70% relative humidity. Adult moths were kept in screen cages and supplied with a 10% sugar solution. Moth populations were refreshed every year with males caught from the wild by means of pheromone traps, as previously described [14]. All females used in this study were 5.5 days old. As a routine, representative of each colony used for histochemical studies, were tested for their capability to synthesize sex pheromone in response to the injection of 1 pmol synthetic PBAN1-33NH$_2$. All tested colonies were positive generating pheromone amounts that ranged from 144 to 189 ng per female.

2.2. Ligands and other peptides

Photo-affinity biotinilated peptide ligands were synthesized as described below. All other peptides were prepared as previously described [48]. The purity of all tested peptides was 90–95%.

2.2.1. Chemicals for peptide synthesis

Fmoc-protected amino acids with standard side-chain protecting groups, Rink amide 4-methylbenzhydrylamine (MBHA) resins and reagents for peptide synthesis were purchased from NOVA Biochem (Switzerland). Ultra-pure quality solvents were purchased from Baker (USA). Other reagents were purchased from Aldrich.

2.2.2. Synthesis of photo-affinity biotinilated ligands

Two biotinilated photo-affinity ligands were synthesized: a biotinilated-photo-affinity full-length PBAN molecule (BpaPBAN1-33NH$_2$) and a shorter fragment derived from its C-terminus Arg^{27}-PBAN28-33NH$_2$ (BpaArg^{27}-PBAN28-33NH$_2$). A *p*-benzoyl-Phe amino acid (Bachem, Switzerland) was substituted for Phe^{29} in each PBAN molecule.

Peptides were synthesized by the solid phase peptide synthesis methodology [9] on a Rink amide MBHA resin (0.6 mmol/g loading), by means of 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Coupling of Fmoc-protected amino acids was performed by using: a three-fold excess of pre-activated amino acid, a three-fold excess of bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBroP), and a seven-fold excess of diisopropylethylamine

(DIEA) in *N*-methylpyrrolidinone (NMP) for 10 min prior to coupling. Each coupling reaction was continued for 3 h. The Fmoc-protecting group was removed with 20% piperidine in NMP. After each coupling and Fmoc deprotection the resin was washed with NMP (5 × 2 min) followed by dichloromethane (DCM) washing (2 × 2 min).

2.2.3. Attachment of biotin to the peptides

After Fmoc deprotection of the last amino acid on the peptidyl resin, a coupling reaction was performed with biotin (Sigma, St. Louis, USA) with a three-fold excess of biotin preactivated with a three-fold excess of PyBroP and a seven-fold excess of DIEA in NMP, for 10 min prior to coupling. The coupling reaction was repeated twice, for 2 h each time. At the end of the reaction the peptidyl resin was washed with NMP (5 × 2 min), DCM (2 × 2 min) and methanol (twice), and the peptidyl resin was dried under vacuum.

2.2.4. Deprotection of side chain groups and cleavage of peptides from the resin

Peptides were deprotected and cleaved from the resin with 90% trifluoroacetic acid (TFA) in the presence of scavengers (1% double-distilled water (DDW) and 1% triisopropylsilane). The cleavage process continued for 3 h at room temperature (RT). The resin was removed by filtration and washed with TFA (2 × 5 ml). The TFA filtrate was evaporated to dryness under a stream of nitrogen. The peptide was precipitated with cold diethyl ether and washed twice with ether. The crude peptide thus obtained was dissolved in 50% acetonitrile (ACN) and lyophilized.

2.2.5. Purification of peptides

The crude peptides were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a Vydac RP-18 column (25 mm × 250 mm) with a Merck-Hitachi 655A liquid chromatography pump. The solvent systems used were ACN and DDW, each containing 0.1% TFA. The peptides were eluted between 15 and 70% ACN in 40 min. The flow rate was maintained at 9 ml/min and the peptides were detected at 220 nm. Purity and homogeneity of the peptides were cross-checked by analytical RP-HPLC on a Merck Lichrocart RP-18 column (5 mm × 250 mm) with a Merck-Hitachi L-700 Lachrom liquid chromatography pump. The flow rate was 1 ml/min and the absorbance was detected at 220 nm. The peptide purity was found to be in the range of 90–95%. Purified peptides were characterized by MALDI time-of-flight-mass spectrometry (TOF-MS, linear CHCA matrix) and amino acid analysis of hydrolizates. The molecular masses obtained were 4219.96 and 1253.61 for BpaPBAN1-33NH$_2$ and BpaArg^{27}PBAN28-33NH$_2$, respectively.

2.3. Determination of the pheromonotropic activity of Bpa peptides

The pheromonotropic activities of Bpa peptides and their corresponding non-biotinilated analogs were determined

in *H. peltigera* females by means of the pheromonotropic bioassay, as previously described [2]. Activity was determined by the injection of a dose of 0.1–1000 pmol of each peptide for 2 h. At the end of the experiment glands were excised and their pheromone contents were determined by capillary gas chromatography as previously described [2].

2.4. Histology

2.4.1. Fixation and embedding of tissue for light microscopy

Fully extended ovipositors of 5.5-day-old *H. peltigera* females were excised at the 5–7 h of photophase and fixed in 2% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS, 0.15 M NaCl in Na phosphate buffer, pH 7.2), for 2 h at RT. Fixed ovipositors were stored in 0.5% PFA at 4 °C until use. Before use, the glands were rinsed briefly with PBS (2 × 5 min), transferred to 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and microwave irradiated (General Electric Model JES 633) at 480 W for 30 s to increase fixation efficiency [41]. Ovipositors were then transferred to 1 ml of fresh 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 and re-fixed for 2 h at RT. At the end of the fixation the tissue was rinsed with PBS, pH 7.2 (6 × 10 min) and post-fixed with 2% (v/v) osmium tetroxide in DDW for 2 h at RT. The tissue was then rinsed (3 × 30 min) with DDW and subjected to a series of dehydration steps comprising soaking in increasing percentages of acetone in DDW (30% for 60 min; 50% for 60 min; 70% for overnight (ON); 95% for 60 min; 100% for 3 × 60 min). The tissue was then embedded in Spurr (Ted Pella, Redding, CA) according to the following protocol: 2:1 (acetone:Spurr) for 4 h at RT with shaking; 1:2 (acetone:Spurr) ON with very slow shaking, and 5 × 4 h of straight Spurr. The embedded tissue was transferred to rubber molds in straight Spurr, positioned to facilitate coronal or sagittal sectioning, and allowed to polymerize for 18–20 h at 60 °C.

2.4.2. Tissue sectioning

Three 1 μm coronal sections were collected every 20 μm and stained with methylene blue. A total of 50 tissue samples representing different areas of the ISM and the 9th abdominal segment were examined.

2.5. Scanning electron microscopy (SEM)

Fully extended ovipositors of 5.5-day-old *H. peltigera* females were excised at the 5–7 h of photophase. Tissue was fixed and dehydrated in acetone as above (Section 2.4.1). Ovipositors were attached to a copper specimen holder with cryo-adhesive tape and critical point dried. At the end of the process, the tissue was coated with gold palladium at 15 mA/min for 3 min, and viewed with an SEM (Phillips XL-30 FEG) as previously described [27].

2.6. Histochemistry

2.6.1. Fixation of tissue for frozen sections

Fully extended ovipositors of 5.5-day-old *H. peltigera* females were excised at the 5–7 h of photophase, fixed in 2% PFA in PBS for 2 h at RT and stored in 0.5% PFA at 4 °C until use. Before use, the ovipositors were washed briefly in PBS (2 × 5 min), re-fixed in 4% PFA in PBS for 30 min at RT, washed with PBS (3 × 15 min) and transferred to 0.1 M glycine in PBS for 30 min (to block free aldehyde groups). The tissue was then washed with PBS (2 × 10 min) and transferred to 15% (w/v) sucrose solution in PBS at 4 °C with gentle shaking until the tissue sank (2–4 h). Next, the tissue was transferred to 30% (w/v) sucrose solution in PBS and was kept ON at 4 °C. On the next day, the tissue was removed from the sucrose solution, transferred to plastic molds, dried from all remaining sucrose solution and mounted (in the desired position) with cold (4 °C) OCT (Triangle Biomedical Sciences, Durham, NC). The molds were then placed on dry ice until the OCT froze, and were stored at −70 °C until further use.

2.6.2. Preparation of frozen sections

Coronal sections, 10 μm thick, were cut with a Microm HM500 OM cryostat. Sections were placed on gelatin-coated slides and kept at −70 °C until further use.

2.6.3. Receptor localization

Sections were immersed in PBS (2 × 10 min) to remove the OCT, and were re-fixed in 4% PFA in PBS for 15 min at RT. Excess PFA was washed off with PBS (2 × 5 min) and the sections were transferred to 0.1 M glycine in PBS for 20 min as above (to block reactive groups). After another rinse with PBS (10 min) the tissue was incubated with avidin (Sigma) in PBS at 0.1 mg/ml (30 min at RT) (to block endogenous biotin). Unbound avidin was washed away with PBS (4 × 5 min) and the sections were incubated with biotin (Sigma) in PBS at 0.01 mg/ml (30 min at RT) to block free avidin sites. Unbound biotin was washed away with PBS (4 × 5 min) and the sections received an additional rinse (5 min) with reaction buffer (10 mM NaHCO$_3$, 145 mM sucrose, 10 mM HEPES, pH 8.0). The sections were then incubated for 75 min at RT with 10 pmol of ligand (BpaPBAN1-33NH$_2$ or BpaArg^{27}-PBAN28-33NH$_2$) in the presence or absence of 6 nmol of competing peptides (PBAN1-33NH$_2$ or PBAN28-33NH$_2$) made up in reaction buffer. At the end of the incubation the sections were irradiated for 30 min at 4 °C with a 2 × 15 W 375 nm UV lamp placed at a distance of 9 cm from the reaction mixture. Then the sections were rinsed with PBS (4 × 5 min) at RT, transferred to a blocking solutions of 0.5% (w/v) bovine serum albumin (BSA) in PBS for 20 min and incubated with streptavidin fluorescein (FITC) or streptavidin Alexa Fluor 568 conjugates (Molecular Probes, Eugene, OR) (diluted 1:500) in 0.3 M NaCl made up in 50 mM Na phosphate, pH 7.2 for 60 min in the dark. Unbound reagents were washed away with PBS (4 × 5 min)
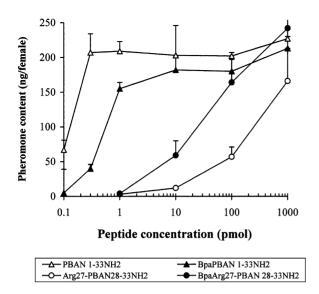
Fig. 1. Dose–response curve of pheromonotropic activity elicited by photo-affinity biotinilated (Bpa) and non-biotinilated PBAN1-33NH$_2$ and Arg^{27}PBAN28-33NH$_2$. Peptides were injected at the indicated concentrations for 2 h. Injection of buffer was carried out similarly and served as a control. Pheromone content was determined by capillary gas chromatography analysis. Control values ranged from 0 to 20 ng per females. Each value represents the mean ± S.E.M. of 8–10 samples.

and nuclei were stained with the nuclear dye TO-PRO-3 iodide (Molecular Probes) (diluted 1:1000 in PBS containing 0.1% Triton-X-100) for 15 min. Excess dye was washed away with PBS (3 × 5 min), the slides were dried and the sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The specimens were stored at 4 °C in the dark. Microscopic examination was performed with a laser confocal microscope (Zeiss LSM510). A minimum of three specimens, representing at least two different moth generations, was tested for ligand binding.

3. Results

A preliminary requirement for receptor localization studies is the availability of a highly potent ligand. For that purpose we synthesized photo-affinity ligands that could bind covalently to the receptor. Two biotinilated photo-affinity ligands (BpaPBAN1-33NH$_2$ and BpaArg^{27}-PBAN28-33NH$_2$), were synthesized, in which a *p*-benzoyl-Phe amino acid was substituted for a Phe^{29} in each PBAN molecule. In order to determine whether the substitution of the Phe^{29} and addition of a biotin residue to the molecule affected activity, both Bpa ligands were tested for their in vivo pheromonotropic activity and compared with that of the parent molecules. As indicated in Fig. 1, BpaPBAN1-33NH$_2$ exhibited a slightly lower activity than the parent non-biotinilated peptide although at a concentration of 10 pmol and above the activity was comparable with that of PBAN1-33NH$_2$. BpaArg^{27}-PBAN28-33NH$_2$, on the

other hand, was more active than Arg^{27}PBAN28-33NH$_2$ at concentrations of 10 pmol and above. The availability of highly bioactive photo-affinity biotinilated peptides enabled us to further characterize the receptor and to localize it in the ovipositor of *H. peltigera* by means of streptavidin-coupled fluorophore reporters.

A secondary requirement for histochemical studies is elucidation of the structural details of the tested tissue. For that purpose we excised ovipositors of female *H. peltigera* moths and performed a detailed analysis of the pheromone gland structure by light microscopy (LM) and scanning electron microscopy (SEM). For the LM study we prepared 1 μm sagittal sections of various regions of the ovipositor as well as 1 μm coronal sections (at 20 μm intervals) of the entire ovipositor (regions I through III in Fig. 2A). Examination of the intact ovipositor under a stereoscopic microscope revealed an ISM (region II Fig. 2B and C) bordered by two heavily sclerotized cuticular regions that formed the 9th and 8th abdominal segments (regions I and III, respectively, Fig. 2B and C). The heavy sclerotized cuticle of the 8th segment formed an almost complete ring whereas that of the 9th abdominal segment formed two sclerotized valves that extended around the segment laterally but did not completely meet one another ventrally (Fig. 2C) or dorsally (not shown).

LM examination of the stained coronal and sagittal sections revealed that the pheromone gland comprised the ISM (region II in Fig. 2A–C) between the 9th and 8th abdominal segments. The pheromone gland cells comprised a single layer of columnar epithelial cells positioned under the inter-segmental cuticle (Fig. 2D and E). These cells extended along the entire ISM and surrounded the entire circumference of the tissue, to form a complete ring-type gland (Fig. 2F). Epithelial cells, similar to those of the ISM, extended all the way to the tip of the ovipositor. Fig. 2G and H depict the ventral and dorsal epithelial layers (regions V and D) overlaid by the inter-segmental cuticle (light blue color, Fig. 2G) and the heavily sclerotized lateral cuticular valves of the 9th segment (dark blue color Fig. 2G). SEM studies revealed that the pheromone gland region is highly convoluted (Fig. 3A–C), densely covered with hollow cuticular spines (Fig. 3D–F) that cover the entire circumference of the ISM, and through which the pheromone is, most likely, secreted.

In order to localize PK/PBAN receptor-containing cells, ovipositors were fixed, and slide-mounted 10 μm frozen sections were incubated with the Bpa ligands, UV irradiated (for covalent attachment of the ligand to the tissue), and visualized by application of streptavidin conjugated to an Alexa or FITC fluorophore. Binding of BpaPBAN1-33NH$_2$ to the sections revealed that the epithelial cells of the ISM were heavily stained with the ligand (Fig. 4A and B). The BpaPBAN1-33NH$_2$ ligand was fully displaced in the presence of a 600-fold excess of non-biotinilated PBAN1-33NH$_2$ (Fig. 4C) or PBAN28-33NH$_2$ (Fig. 4D). Staining was also obtained with BpaArg^{27}-PBAN28-33NH$_2$ (Fig. 4E) and this ligand was also fully displaced with non-biotinilated
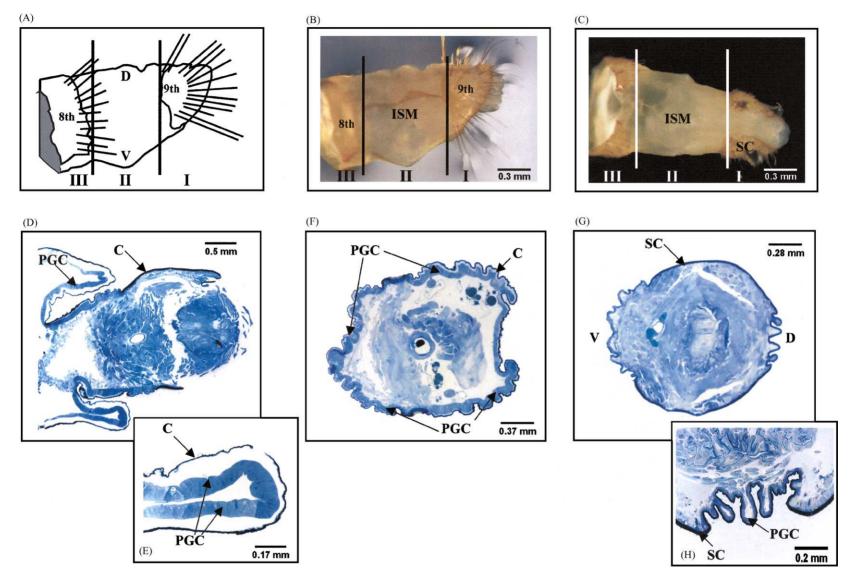
Fig. 2. (A) Schematic representation of a lateral view of an extended ovipositor of a 5.5-day-old *Heliothis peltigera* female. Regions III and I indicate the 8th and the 9th abdominal segments, respectively. Region II indicates the inter-segmental membrane (ISM) that forms the pheromone gland. (B) Stereo microscope picture of a lateral view of an extended ovipositor (4×). Regions I, II and III correspond to those in (A). (C) A ventral view of an extended ovipositor (4×) showing epithelial cells enclosed between a heavily sclerotized cuticle (SC) of the 9th segment. (D) Sagittal light microcopy (LM) section through partially retracted ovipositor (10×). (E) Higher magnification (40×) of pheromone gland cells (PGC) in (D) showing large columnar epithelial cells. (F) Coronal LM section through the pheromone gland region (II in A–C) (10×) showing a continuous ring of glandular cells. (G) Coronal LM section through the 9th abdominal region (I in A–C) (10×) showing the convoluted dorsal and ventral regions as well as the smooth heavily sclerotized cuticle of the 9th segment. (H) Higher magnification (40×) of the convoluted dorsal area enclosed between the sclerotized cuticle of the 9th segment. Sections in (F) and (G) represent a series of coronal sections of the inter-segmental and 9th segment regions, respectively, all of which exhibit a similar structure. Stereomicroscopic images were obtained with a Nikon SMZ-U Microscope using ovipositors fixed in 2% formaldehyde in PBS for 2 h at RT. LM images were obtained with 1 μm sections embedded in Spurr as described in Section 2.4; C: cuticle; D: dorsal part of ovipositor; V: ventral part of ovipositor.
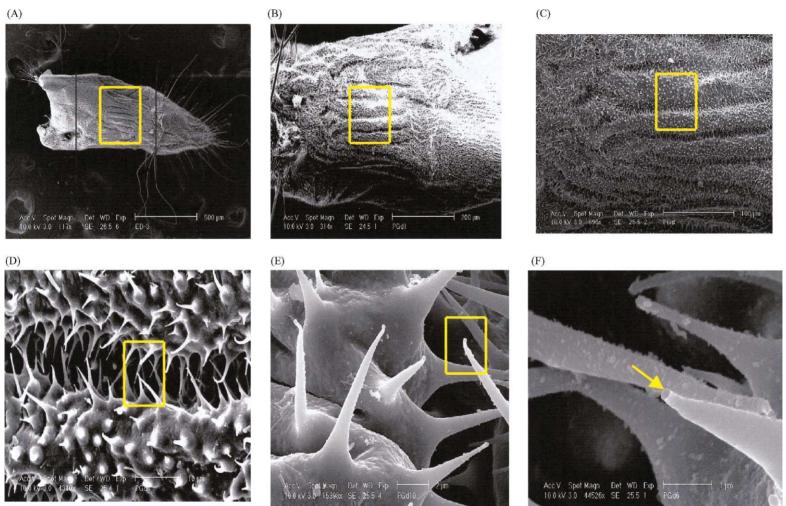
Fig. 3. (A) Scanning electron microscopy (SEM) images of the surface of an extended ovipositor (5.5-day-old *H. peltigera* female). (B–F) Dorsal view showing convoluted areas of the gland surface and hollow cuticular hair (arrow). Yellow squares mark region enlarged in the following image.
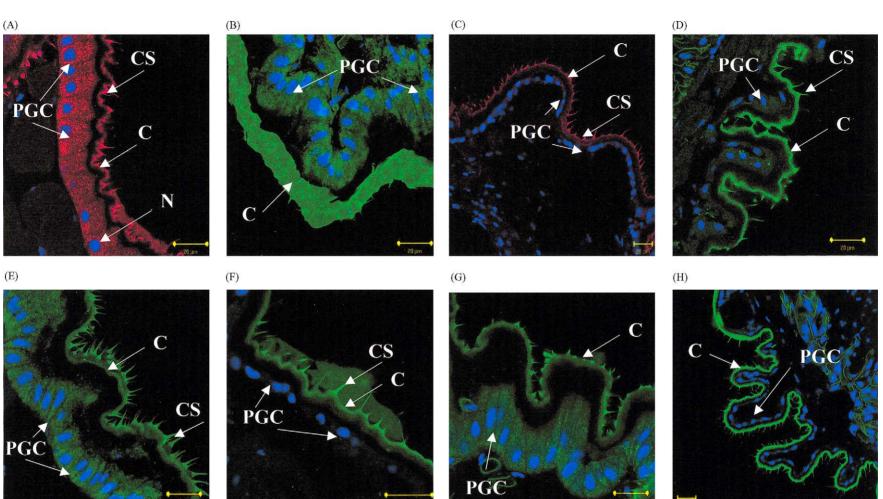
Fig. 4. Confocal microscopy (CM) images showing PK/PBAN receptor containing cells in the ISM of *H. peltigera* ovipositor. PBAN-containing cells were visualized with streptavidin conjugated to Alexa (A, C) or FITC (B, D, E–H). (A) Binding of BpaPBAN1-33NH$_2$ stained with sterptavidin Alexa conjugate (100×); (B) binding of BpaPBAN1-33NH$_2$ stained with streptavidin FITC conjugate (100×); (C) binding of BpaPBAN1-33NH$_2$ in the presence of a 600-fold excess of non-biotinilated PBAN1-33NH$_2$ (100×); (D) same in the presence of a 600-fold excess of non-biotinilated PBAN28-33NH$_2$ (100×); (E) binding of BpaArg^{27}-PBAN28-33NH$_2$ (100×); (F) displacement of BpaArg^{27}-PBAN28-33NH$_2$ with PBAN28-33NH$_2$ (100×); (G) displacement of BpaArg^{27}-PBAN28-33NH$_2$ with PBAN1-33COOH (100×); (H) control. Non-specific adsorption of streptavidin FITC conjugate to the tissue in the absence of any ligand (40×); blue staining represents nuclei stained with To-PRO 3-iodide. C: cuticle; CS: cuticular spines; N: nuclei; PGC: pheromone gland cells. Bar represents 20 μm. Each image represents data obtained in two to four independent experiments.
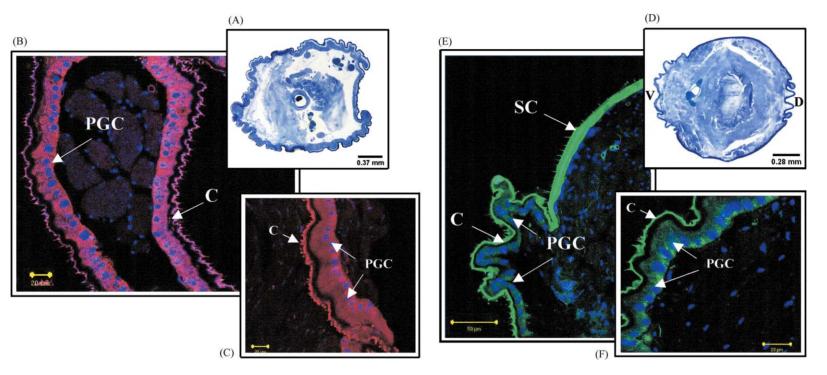
Fig. 5. CM images showing PK/PBAN receptor-containing cells in different regions of the ovipositor (regions I and II in Fig. 2A–C). (A) Coronal LM section through the ISM region (10×). (B) CM image (40×) showing presence of PBAN receptor containing cells in the entire circumference of the tissue. (C) Enlargement of the above PBAN receptor containing cells, showing the basal location of the receptors in the PGC. (D) Coronal LM section through the 9th abdominal region (10×). (E) PBAN receptor-containing cells in the ventral parts of the 9th abdominal segment region of the ovipositor (40×) showing PBAN-containing cells in the region underneath the "thin" cuticle and absence of staining in cells underlying the sclerotized cuticle (SC). (F) Higher magnification (100×) of the PBAN receptor-containing cells in (E), showing the basal location of the receptors. C: cuticle; D: dorsal part of ovipositor; PGC: pheromone gland cells; V: ventral part of ovipositor. Bars in (C), (D) and (F) represent 20 μm. Bar in (E) represents 50 μm.

PBAN28-33NH$_2$ (Fig. 4F). The C-terminally free acid PBAN analog (PBAN1-33COOH) that is devoid of any pheromonotropic activity [2], did not displace the ligand (Fig. 4G). Also, the staining was highly specific and no binding of streptavidin–fluorophore conjugates could be demonstrated in the absence of ligands (Fig. 4H). The peripheral heavy red or green stain that appears in all sections was contributed by cuticular auto-fluorescence, which was also prominent in the absence of any ligand or in the presence of an excess of non-biotinilated PBAN1-33NH$_2$ or PBAN28-33NH$_2$ molecules.

Next, we set out to determine the spatial distribution of the receptor within the pheromone gland. For that purpose, the whole ovipositor (regions I through III in Fig. 2A) was sectioned into 10 μm sections and sets of three serial sections were stained sequentially with BpaPBAN1-33NH$_2$ and BpaArg^{27}-PBAN28-33NH$_2$ ligands. Throughout region II (represented by Fig. 5A) binding of both ligands stained the entire circumference of the tissue (Fig. 5B), clearly indicating the presence of the receptor in all parts of the ISM. In the 9th segment (represented by Fig. 5D), only the dorsal and ventral regions were stained (Fig. 5E). In the lateral areas, where a heavily sclerotized cuticle was visualized, no staining was observed (Fig. 5E). No cells, other than the glandular cells underneath the "thinner" cuticle, were stained in regions I and II (Fig. 5B, C, E and F), indicating the high specificity of the staining. No staining was observed in region III (8th abdominal segment). Interestingly, the staining in the ISM pheromone gland cells as well as in the glandular cells of the dorsal and ventral parts of the 9th abdominal segments exhibited a polar pattern, with intense staining appearing at the basal part of the epithelial cells (Fig. 5C and F). The patterns obtained with both ligands BpaPBAN1-33NH$_2$ and BpaArg^{27}-PBAN28-33NH$_2$, were similar.

4. Discussion

In the present study we demonstrated the presence of the PK/PBAN receptor in pheromone gland cells of *H. peltigera* females, and visualized its spatial distribution within the ovipositor.

Receptor visualization was performed with two photo-affinity biotinilated ligands: a full-length PBAN1-33NH$_2$ and Arg^{27}-PBAN28-33NH$_2$. Photo-affinity labeling is a very powerful technique for histochemical investigation of receptors, but it is necessary to consider carefully the choice of the peptide and the photo-label, the design of the photo-reactive peptide, and the irradiation process to ensure that the photo-labeled peptide will remain active, that the chemistry of labeling will be relatively simple and highly efficient, and that the photo-ligand will be stable and reactive to a degree that will not cause high non-specific interactions. In light of the above considerations we chose to use benzophenone-substituted peptides that fulfill the above requirements and have previously been successfully

used to label a variety of receptors [44,49]. The PBAN-modified photo-affinity ligands were further biotinilated at the N-terminus in a manner that did not affect their bioactivity and enabled their employment in histochemical studies for visualization of the PK/PBAN receptor with the aid of streptavidin-conjugated fluorophores.

In order to detect PBAN receptor-containing cells among the pheromone producing glandular cells in the ISM and other regions of the ovipositor it was necessary to perform a detailed histological study of the structure of the pheromone gland of *H. peltigera*. Although Noctuidae species have been examined more than any other family and considerable information has accumulated concerning the structure of the cells that form the sex pheromone gland, the extent to which the ISM is glandular is extremely variable among moths.

Our present study indicates that the pheromone gland of the *H. peltigera* female is a simple unicellular ring gland, whose glandular cells encircle the ovipositor, and occupy the entire ISM between the 8th and the 9th abdominal segments. Glandular cells were also found in the dorsal and ventral part of the 9th abdominal segment, laterally divided by a sclerotized cuticular wall. By analogy with findings in other *Heliothinae* species [33], we assume that the cells underneath the sclerotized cuticle are unmodified squamous cells or cells modified for the insertion of muscles. Based on the above it seems that the structure of the pheromone gland of *H. peltigera* is similar to that of two other *Heliothinae* species: *H. zea*, that was found to be ring glands with dorsal and ventral glandular cells in the 9th segment [8,22,33,41] and *H. phloxiphaga* [22].

Despite the information that has been accumulated concerning the structure of the glandular cells, the biochemical elucidation of the structural–functional relationship of these cells is still incomplete. Several studies have tried to correlate the structure of the pheromone gland with its function as a pheromone-producing organ and to define the spatial distribution of pheromone-producing cells. The approach that has been commonly employed to address the issue was based on examination of ultra-structural changes associated with the production and release of pheromone by maturing gland cells. Indeed, it has been found in several studies that the cells change from primarily protein-secreting cells to primarily lipid-secreting cells [33]. Recently, Raina et al. [41] addressed the issue by monitoring pheromone titers in various sections of the *H. zea* ovipositor, correlating them with morphological changes that follow pheromone production. This study led to a much more precise localization of the pheromone-producing regions within the ovipositor and indicated specific cellular changes that occur during pheromone production and non-production periods.

An alternative and more direct approach to the localization of sex pheromone producing cells in the ovipositor is the cellular approach where direct visualization of cells expressing receptors of pheromonotropic peptides (e.g. PK/PBAN) can be obtained. PK/PBAN peptides have long been known to stimulate sex pheromone biosynthesis in moths, thus, a

demonstration of the presence of PK/PBAN receptors on the epithelial cells of the ISM and other regions of the ovipositor provides direct evidence for the presence of pheromone-producing cells.

Two photo-affinity biotinilated ligands were chosen for this purpose: The full-length PBAN (BpaPBAN1-33NH$_2$) which is considered to be the prime pheromonotropic peptide; and a modified C-terminally derived analog Arg^{27}-PBAN28-33NH$_2$ (BpaArg^{27}-PBAN28-33NH$_2$), which contains the "signature sequence" of the PK/PBAN family, and which has been found to elicit pheromone biosynthesis in a manner similar to that of the full-length PBAN [2,48]. The latter ligand is, theoretically, a more "universal" PK/PBAN ligand exhibiting a higher potential than PBAN1-33NH$_2$ to bind to multiple receptors of the PK/PBAN family.

Employment of both ligands on slide-mounted, fixed sections stained the columnar epithelial cells throughout the ISM as well as the ventral and dorsal epithelial cells in the 9th abdominal segment. Epithelial cells underlying the sclerotized cuticle were not stained. The patterns obtained with both ligands: BpaPBAN1-33NH$_2$ and BpaArg^{27}-PBAN28-33NH$_2$ were similar, indicating two possibilities: either that both ligands bind to the same receptor under the tested conditions, or, if there are two distinct receptors, that their spatial distribution through out the gland is very similar. Staining exhibited a polar pattern, with intense staining appearing at the basal part of the epithelial cells. This polarity of the PBAN receptor most likely facilitates efficient contact with the hemolymph and the blood-borne hormones (e.g. PBAN) that stimulate sex pheromone production in these cells. Staining with both ligands was highly specific: no other cells in the tissue were stained, binding was fully displaced with an excess of non-biotinilated ligands (PBAN1-33NH$_3$ and PBAN28-33NH$_2$), and was not displaced with the C-terminally free acid analog PBAN1-33COOH, which is devoid of pheromonotropic activity [2]. It is interesting to note that although the glands used in this study were excised at photophase, they still revealed the presence of a marked amount of PK/PBAN receptors. This is in line with previous findings from our laboratory [18] as well as others [36] that moths are responsive to PBAN at photophase, although natural pheromone production occurs during the scotophase.

In summary, our data clearly demonstrate the presence of a PBAN receptor throughout the ISM region as well as in ventral and dorsal region of the 9th abdominal segment, which indicates that the columnar epithelial cells in both regions (ISM and parts of the 9th segment) are pheromone-producing cells. These data correlate well with the findings of all the studies that considered on the basis of morphological criteria, that the columnar cells are pheromone-producing cells, and with the recent data of Raina et al. [41] who demonstrated that about 70% of pheromone-producing cells are present in the ISM (in both the dorsal and ventral region), and that 17% of these cells are present in the 9th segment.

Several authors [33,46] indicated, on the basis of behavioral and microscopic studies, that pheromone is produced in the 9th abdominal segment, but that this region may contribute differently to the composition of the pheromone blend. Our present findings on the presence of PK/PBAN receptor in the 9th abdominal segment clearly support the hypothesis that pheromone is indeed produced in this region, although we are unable to determine whether the nature of pheromone produced in this region is different from that produced in the main ISM area. It should be noted, however, that since both ligands used in our study exhibited a similar pattern throughout the ovipositor it is unlikely to consider that pheromone components that are synthesized in the cells of the 9th segment differ from those synthesized in the ISM region, unless the same ligands activates similar receptors in different areas, leading to the initiation of different biosynthetic pathways.

In addition to the spatial localization our data also clearly indicate that the pheromone gland serves as a target for PBAN. As indicated in Section 1, the issue of the target tissue and transport route of PBAN has engaged, and still engages many research groups dealing with the neuroendocrine control of sex pheromone biosynthesis in moths. Our present study, as well as our previous biochemical and pharmacological study of the PK/PBAN receptor in the pheromone gland of *H. peltigera* [6,7] contribute direct evidence for the target tissue issue although it does not resolve the problem of the transport route. Although we indicated above that the basal location of the receptors on the epithelial cells hints at the possibility that the receptors are arranged so as to ensure them an easier access to humoral factors (which would indicate a hemolymph-borne factor as the stimulator of pheromone biosynthesis), it is still possible that nerve cells terminating on the epithelial glandular cells stimulate the biosynthetic process. Unfortunately, our study did not focus on this issue and the presence of such nerve terminals was not visualized.

Another issue that still needs to be further addressed is that of the involvement of additional factors in the stimulation of sex pheromone biosynthesis in the gland. Despite our clear cut data on the presence of PK/PBAN receptors in the gland (which strongly indicate the role of these peptides in this process) the possibility that other factors acting directly or indirectly on the gland, cannot be excluded and needs further investigation.

To the best of our knowledge this is the first report on the histochemical visualization of the PK/PBAN receptor in the pheromone gland. The ligands that are described in this study as well as the histochemical methods that were employed to visualize the receptor could serve as a basis for the localization of receptors belonging to this family in the pheromone glands of other moth species as well as in other organs.

Acknowledgments

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