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PBAN receptor: Employment of anti-receptor antibodies for its characterization and for development of a microplate binding assay☆

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ABSTRACT

This study describes generation of an anti-PBAN receptor (PBAN-R) antiserum and its employment for the characterization of the PK/PBAN-R(s). The antiserum recognized, in a specific and dose-dependent manner, the presence of PBAN-R in pheromone gland membrane preparations of three female moths: *Heliothis peltigera*, *Helicoverpa armigera* and *Spodoptera littoralis*. It also reacted specifically with the *S. littoralis* larval receptor *in vivo*, most likely by competing with the ligand on the binding site and consequently inhibiting cuticular melanization. Despite its ability to react with the receptor of *H. peltigera* in dot blot experiments, the antiserum did not react with the receptor *in vivo* and failed to inhibit sex pheromone biosynthesis. The antiserum was also used to develop two microplate binding assays. The Ab described in this study is the first raised against an insect neuropeptide (Np) receptor to be used *in vivo*, and its employment for characterization of the PK/PBAN-R(s) may thus provide important information on the mode of action of this Np family. The present study adds important information on the difference between the receptors in the two moth species, hints at the possible existence of receptor subtypes, and provides a platform for the development of a high-throughput assay (HTA) for screening of PK/PBAN agonists and antagonists.

1. Introduction

Pheromone biosynthesis activating neuropeptide (PBAN) was first reported by Raina and Klun (1984) as the neuropeptide (Np) that regulates sex pheromone production in female moths (*Helicoverpa zea*). PBAN was isolated from *H. zea* in 1989; it was characterized as a 33-amino-acid C-terminally amidated Np and was designated as Hez-PBAN (Raina et al., 1989). Since 1989 the primary sequences of 19 PBAN molecules have been determined in many other moth species, and all of them were found to share a common C-terminal pentapeptide sequence: FSPRLa. Further studies based on molecular cloning have revealed that the sequence of the cDNA encoding the PBAN pre-prohormone also encodes four other peptides, in addition to PBAN, which have the PBAN consensus sequence FXPRLa (X = S, T, G or V): diapause hormone (DH), two subesophageal ganglion Nps (SGNP β and γ), and one FXPKLa peptide (α-SGNP). The gene that encodes these five peptides was termed the DH-PBAN gene (for a detailed review see (Altstein and Hariton, in press)).

Advances in insect Np studies revealed that other peptides, which do not originate from the DH-PBAN prohormone, also have the same signature sequence. They include: the PKs (Lem-PK also termed LPK, Lom-PK-I and Lom-PK-II); the myotropins (Lom-MT-I to IV), which are myotropic peptides isolated from the cockroach *Leucophaea maderae* and the migratory locust, *Locusta migratoria* (Nachman et al., 1986; Nachman and Holman, 1991; Schoofs et al., 1991, 1993); and a myotropic peptide from *Schistocerca gregaria* (Scg-MT-1) (Veelaert et al., 1997). Additional peptides that were found to share the same consensus sequence are: pheromonotropin (Pss-PT), an 18-amino-acid peptide isolated from *Pseudaletia* (*Mythimna*) *separata* (Matsumoto et al., 1992) that shares high sequence similarity with β-SGNP; peptides from *D. melanogaster* – Drm-PK-1 (Kean et al., 2002) and Drm-PK-2 (Meng et al., 2002) – each of which is encoded by a different gene (Olsen et al., 2007); and two PK peptides from *Periplaneta americana* – Pea-PK-5 and Pea-PK-6 – (Predel et al., 1999; Predel and Eckert, 2000). All peptides sharing the above sequences were grouped into one family, designated the FXPR(K)La family or the PK/PBAN family, named after PBAN and Lem-PK, which was the first member of this family to be identified (Holman et al., 1986). The signature sequence of the family (FXPRLa; X = S, T, V or G) was found to represent the minimal sequence required for induction of pheromonotropic activity, and under certain conditions the C-terminal hexapeptide of PBAN (YFSPRLa) exhibited a biological activity indistinguishable from that of the full-length peptide (Altstein et al., 1995 and references therein).

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Studies performed in many laboratories, including ours, indicated that the PK/PBAN peptide family, currently known to comprise over 30 Nps, is a ubiquitous multifunctional family that plays a major physiological role in regulating a wide range of developmental processes in insects: pupariation (Nachman et al., 1997); diapause (Imai et al., 1991); cuticular melanization (Altstein et al., 1996; Matsumoto et al., 1990); feeding, i.e., gut muscle contraction; and mating behavior, i.e., sex pheromone production. The involvement of PK/PBAN Nps in the above functions was demonstrated by means of a variety of *in vivo* and *in vitro* bioassays (pheromonotropic, melanotropic, egg and pupal diapause, pupariation and myotropic assays) that were developed and optimized in several laboratories (for review see Altstein and Hariton, in press, and references therein), and the role of the PK/PBAN peptides was determined, in most cases, by injecting synthetic peptides and monitoring the level of activity they elicited. These studies showed that all of the above functions could be stimulated by more than one peptide of the PK/PBAN family, and that the peptides do not exhibit species specificity (for review see Gade, 1997).

The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these Nps elicit their effects such as: Do all peptides mediate a given function in a given insect species by one receptor or by different receptors or receptor subtypes? Are different functions in a given insect species mediated by the same receptor or by different receptor subtypes? Is a given function in different insect species mediated by the same or different receptors? Since the activity of the PK/PBAN peptides was evaluated mainly through employment of synthetic peptides injected *in vivo*, an approach that may not fully reflect their endogenous (native) role, our understanding of the modes of action of this family at the cellular level, of the nature of the native (endogenous) peptides that mediate each function, and of the receptors that mediate them in moths and other insects is very limited and requires further investigation.

One way to shed light on some of these questions is by the use of selective agonists and antagonists. In the past few years we have devised a novel integrated approach, designated Insect Np Antagonist Insecticide, INAI – based on rational design – which has led to the discovery of a few highly potent, conformationally constrained, selective, metabolically stable and bioavailable antagonists (Altstein et al., 1999; Nachman et al., 2009c; Zeltser et al., 2000, 2001). Employment of these compounds in several *in vivo* bioassays hinted at the possibility that different functions are mediated by different receptor subtypes (Altstein et al., 2007).

Another approach to resolve these questions is by studies of the receptor itself. Indeed, in the past few years, extensive studies on the PK/PBAN-Rs have been carried out by means of a variety of techniques (biochemical, molecular, physiological, pharmacological, immunochemical and histochemical) to localize, isolate, clone and characterize the PK/PBAN-Rs in various insects. To date, 12 PK/PBAN-Rs from six insects (mainly moths) were cloned and another five annotated, all of them G-protein-coupled receptors (GPCRs). The receptors were characterized with respect to their gene structure, sequence similarity with other cloned PK/PBAN-Rs, tissue distribution, and ability to bind FXPRLa or PRLa peptides. However, the great majority of the receptors were cloned from whole insects and not specifically from the organs that mediate given PK/PBAN functions (with the exception of the pheromone gland receptors from *H. zea* (Choi et al., 2003), *H. virescens* (Kim et al., 2008) and *B. mori* (Hull et al., 2004)), and only a few detailed pharmacokinetic studies were carried out with these receptors (Cazzamali et al., 2005; Homma et al., 2006; Rosenkilde et al., 2003). Thus, although these approaches provided a large amount of information on the PK/PBAN-Rs they did not enable us to assess the SAR of a given receptor that mediates a given function, or to determine differences in the pharmacokinetic properties of these receptors—information that is essential for resolving the issue of whether receptor subtypes of multifunctional peptide families do indeed exist.

In the present study we generated and characterized a highly specific anti-PBAN receptor (PBAN-R) antiserum and employed it for characterization of the PK/PBAN receptors that mediate sex pheromone biosynthesis in *H. peltigera* adult female moths and cuticular melanization in *S. littoralis* larvae. The study also employed the antiserum for development of a 96-well microplate receptor binding assay which can be further employed for detailed pharmacokinetic studies. Abs are powerful tools for characterizing receptors and were employed in many studies to characterize mammalian GPCRs. The Ab described in the present paper is the first to be raised against an insect Np receptor to be used *in vivo*, and its use for characterization of the PK/PBAN-Rs may thus provide important information on the mode of action of this Np family. The present study gained important information on the differences between the receptors in the two moth species and provides a platform for the development of the microplate binding assay into a high-throughput assay (HTA) for screening agonists and antagonists obtained from natural sources or libraries of synthetic or genetically engineered compounds, in order to identify new control agents based on PK/PBAN-R specific Np antagonists.

2. Materials and methods

2.1. Antibody preparation

2.1.1. Hapten design, synthesis and purity analysis

A peptide corresponding to amino acids 21-35 of the N-terminal part of the *H. zea* pheromone gland, PBAN-R TNVTSHPAAYDEQYA (PBAN-R-21-35), was chosen for generation of the polyclonal antibodies (Pabs). The peptide was synthesized by Invitrogen (San Diego, CA) and partially purified on a reverse-phase C18 Sep-Pak cartridge column (Waters, Milford, MA) as previously described (Gazit et al., 1990). The peptide eluted from the column was tested for purity on a YMC-Pack ODS-AM column (250 mm × 4.6 mm, S-5 μm particle size, 120 Å pore size) on a Agilent 1100 HPLC. Detection was at 214 nm at ambient temperature. Solvent A = 10% acetonitrile (ACN) containing 90% 0.08 M ammonium phosphate buffer, pH 4.5; solvent B = 73% ACN containing 27% 0.08 M ammonium phosphate buffer, pH 4.5. The diluent of all of the above solutions was 0.1% trifluoroacetic acid (TFA) in HPLC H_2O. Conditions: the sample (10 μl) was applied in 0.1% TFA; the solvent was initially 100% A, programmed to 100% B over 30 min, at a flow rate of 1.1 ml/min. The purity of the peptides was found to be >95%.

2.1.2. Synthesis of a hapten–protein conjugate for injection

PBAN-R-21-35 (hapten) was conjugated to the protein carrier (succinylated Keyhole limpet hemocyanine, KLH, Sigma) via the primary amine of the peptide. The reaction was carried out as follows: 2.5 mg succinylated KLH were dissolved in 1.25 ml of 6 mM sodium phosphate buffer, pH 6.5 (buffer 1), and 100 μl (2.5 mg) of freshly prepared 1-ethyl-3-[3-dimethlaminopropyl] carbodiimide hydrochloride (EDC) were added drop-wise to the succinylated KLH. The mixture was incubated for 60 min at room temperature, and then 1.25 ml (2.5 mg) of PBAN-R-21-35 dissolved in buffer 1 were added. The reaction was incubated for 4 h at room temperature and then overnight (ON) at 4 °C. At the end of the overnight incubation the reaction product was subjected to dialysis against double-distilled water (DDW) for 72 h and was divided into 0.5-ml aliquots for injection.

2.1.3. Injection and bleeds

Antiserum against PBAN-R-21-35:KLH conjugate was raised in rabbits. Two rabbits were used for immunization. Preimmune (PI) serum was drawn prior to the first injection and served as a control for serum specificity. Four injections were administered at 20-day intervals and two bleeds – Pab1 and Pab2 – were obtained, which were collected 50 and 70 days, respectively, after the initial immunization. Antisera were aliquoted and stored at −80 °C.

2.2. Antisera characterization

Antisera characterization was carried out by ELISA. Since the hapten is too small to adsorb to the microplate effectively it was necessary to conjugate it to a carrier molecule that would enable its efficient adsorption. Biotinylation was chosen as the preferred method.

2.2.1. Synthesis of biotinylated hapten

PBAN-R-21-35 was dissolved in phosphate buffer saline (50 mM NaH_2PO_4 containing 150 mM NaCl, pH 7.2, PBS) at a concentration of 1 mg/ml and 1 ml was mixed with 0.15 ml of freshly prepared EZ-Link sulfo-NHS-LC-Biotin (which binds to primary amines at pH 7–9, Pierce, Rockford, IL), dissolved in PBS at a concentration of 1 mg/ml. The biotinylation reagent and the hapten were mixed at a molar ratio of 1:2, respectively. The mixture was incubated for 30 min at room temperature, after which the biotinylated hapten (Bio-hapten) was aliquoted and stored at −20 °C.

2.2.2. ELISA development

Microplates (96-wells, Nunc MaxiSorp, Roskilde, Denmark) were coated with 100 μl (1 μg) of avidin (from egg white affinity purified, Sigma) in 0.05 M carbonate buffer (CB), pH 9.6, and incubated ON at 4 °C. After the ON incubation, wells were washed three times with PBS containing 0.1% Tween-20 (PBST), and 100 μl of Bio-hapten, diluted 1:4000 in PBS were added to the wells and incubated for 1 h at room temperature. Wells were washed again as above, and 150 μl of 0.01% low fat milk diluted in PBS were added, to block non-specific binding sites. The plates were kept at room temperature for 1 h and washed again with PBST as above. Next, 100 μl of six serial dilutions (ranging from 1:1000 to 1:32,000) of PI serum or each of the two antisera (Pab1 and Pab2) in PBS, were added to the wells. Plates were incubated ON at 4 °C, washed as above, and 100 μl of goat anti-rabbit conjugated to horseradish peroxidase (HRP whole molecule, affinity isolated) (Sigma), diluted 1:40,000 in PBST, were added. Plates were incubated at room temperature in a light-proof box on an orbital shaker operating at 150 rpm for 1 h. At the end of the incubation, the microplates were rinsed with PBST as above, and 100 μl of 1-Step Ultra TMB-ELISA Substrate (Pierce, Rockford, IL) were added to the wells. The reaction was stopped after 10–20 min by the addition of 50 μl of 4 M sulfuric acid. The absorbance was monitored with an ELISA reader (Multiscan Multisoft Microplate Reader; Labsystems) at 450 nm. The above experiments revealed that the second bleed (Pab2) was much more potent than the first (Pab1), and that Pba2 diluted at 1:10,000 generated a signal that was high enough to be used for further characterization of the antisera (Fig. 1). The PI serum did not contain any Ab that could recognize the PBAN-R-21-35 hapten (Fig. 1). In light of the above data Pab2 was chosen for all further studies.

2.2.3. Cross-reactivity analysis

The ability of the antiserum to recognize the peptides against which it had been generated (PBAN-R-21-35) as well as PBAN1-33NH_2 and PBAN28-33NH_2 (Altstein et al., 1995) was determined by the above ELISA. Microplates were coated with avidin, the Bio-peptide was adsorbed onto them, and they were blocked against non-specific binding, as described above. Next, 50 μl of 12 serial dilutions of each of the tested peptides in PBS (ranging from 0.001 to 300 ng/well) were added to the wells, followed by addition of 50 μl of antiserum diluted in PBS at 1:5000. In addition to the above samples each microplate contained a set of six wells which determined the maximal binding in the absence of any competing analyte (designated as 100% binding). Another set of six wells (coated with only the avidin without the Bio-peptide, and designated as the assay background samples) served to determine the non-specific binding of the reaction components to the microplate and to each other. Plates were incubated ON at 4 °C, washed, and incubated with 100 μl of goat anti-rabbit conjugated to HRP followed by addition of substrate, as above.

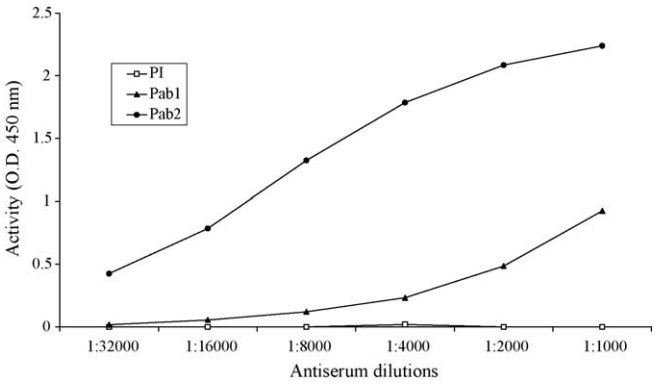

Fig. 1. Dose–response curve of PBAN-R antiserum. PI, preimmune serum: Pab1, first bleed (collected 50 days after initial immunization and two boosts); Pab2, second bleed (collected 70 days after initial immunization and three boosts).

The ability of the antiserum to recognize biotinylated photo-affinity PBAN molecules (Bpa-PBAN1-33NH_2 and Bpa-ArgPBAN28-33NH_2, Altstein et al., 2003) was determined similarly, except that after addition of the Bio-peptide 150 μl (14.36 μg) of biotin (Sigma) dissolved in PBS were added to the wells to block free avidin-binding sites on the plate. Plates were incubated for 1 h at room temperature, washed with PBST and blocked with milk as above. Twelve serial dilutions (50 μl, ranging from 0.03 to 1000 pmol) in PBS were added to the plates, together with 50 μl of antiserum (diluted 1:5000 in PBS) and incubated ON at 4 °C. The plates were washed and further processed as above.

2.3. Preparation of pheromone gland membranes

2.3.1. Insects

H. peltigera moths were reared on an artificial diet as described previously (Dunkelblum and Kehat, 1989). Pupae were sexed, and females and males were placed in separate rooms with a dark/light regime of 10:14 h, at 25 ± 2 °C and 60–70% relative humidity. Adult moths were kept in screen cages and supplied with a 10% sugar solution.

H. armigera were reared on an artificial diet of Instant Soybean Wheat Germ Insect Diet (Stonefly Industries, Bryan, TX) made up according to the manufacturer's recommendations. The moths were reared individually as described for *H. peltigera*, above.

S. littoralis larvae were kept in groups of 100–200 insects in plastic containers (40 cm × 30 cm × 20 cm). Sawdust was placed at the bottom of the container and the top was covered with cheesecloth. The larvae were fed on castor bean leaves and kept in a thermostatically regulated room at 25 ± 2 °C with a light:dark regime of 14:10 h and 60% relative humidity.

2.3.2. Membrane preparation

Pheromone glands were excised from 3.5- to 5.5-day-old *H. peltigera* pheromone glands at the 8th–10th hour of photophase, and kept at −80 °C until use. One day before the experiment, the glands were transferred to a glass–glass homogenizer containing 25 mM HEPES and a commercial protease inhibitor cocktail, Complete Mini EDTA Free (Roche Molecular Biochemicals, Mannheim, Germany) at one tablet per 10 ml of buffer, pH 6.5. Homogenization was performed at a ratio of 10 glands/ml of buffer. The homogenate was centrifuged at 15,000 × *g* in a fixed-angle rotor at 4 °C for 60 min, the supernatant was discarded, and the pellet was re-suspended in DDW. Centrifugation was repeated twice and the resulting pellet was kept overnight at −20 °C. On the next day the pellet (hereafter termed receptor preparation) was re-suspended at a ratio of 10 glands/ml in PBS (unless otherwise indicated) and tested for Ab- or ligand-binding activity. Protein content was determined against a BSA standard curve according to Bradford (1976). Protein concentration ranged from 1 to 4 μg/gland. Pheromone gland receptor preparations of other insects (*H. armigera* and *S. littoralis*), at 3.5–5.5 days of age were carried out similarly.

2.4. Dot blot

This assay served to monitor the ability of the antiserum to recognize the intact pheromone gland PBAN receptor. PVDF filters (Westran Clear Signal, 0.45 μm, 10 cm × 8 cm) (Schleicher and Schuell, BioScience) were pretreated by immersion for 15 s in absolute methanol, followed by a 2-min wash in DDW and a 5-min wash in PBS. Whatman GF/C filters (10 cm × 8 cm) (Tamar, Jerusalem, Israel) were immersed for 5 min in PBS, then were place on top of each other in a Dot Blot apparatus (Bio-Dot, Bio-Rad, Hercules, CA), and subjected to vacuum for 2 min to dry the solvents out. Receptor preparations (100 μl in DDW, equivalent to 1 gland unless otherwise indicated) were applied to the filters for 1 h at room temperature, after which vacuum was applied and the filters were rinsed three times with PBS. In some experiments albumin (Grade VI Ovalbumin from chicken egg white) (Sigma) at an amount corresponding to that of the receptor was added to the wells to monitor non-specific binding of the antiserum to the filter or to other reaction components. Next, 150 μl of 0.1% skim milk powder (Sigma) diluted in PBS were added to the wells to block non-specific binding sites, and the filters were incubated for 2 h at room temperature, after which they were rinsed with PBST and dried under vacuum. Antiserum or PI serum (100 μl diluted in PBS) was added to the wells for 1 h at the end of which the wells were rinsed and vacuum dried as above, and 100 μl of a goat anti-rabbit conjugated to HRP and diluted in PBS (Sigma) was added for one more hour. The wells were rinsed and dried as above; the PVDF filter was removed from the apparatus and transferred to 10 ml of SuperSignal Westdura chemiluminescent substrate (Pierce, Rockford, IL). The reaction was monitored at 1-min intervals for 10–20 min with an Alpha Innotech imager (Model 8800, San Leandro, CA).

2.5. Microplate binding assay

Two assay formats were developed. In the first (format I) PBAN-R Abs were adsorbed to the microplate via protein A. The receptor was separately pre-incubated with covalently bound photo-affinity biotinylated ligand (Bpa-PBAN1-33NH_2), and was added to the adsorbed Abs. The extent of ligand binding was determined by avidin-HRP, with a colorimetric substrate. Non-specific binding was determined in the absence of the biotinylated ligand. In the second format (format II) the biotinylated photo-affinity ligand was adsorbed to the microplates via avidin, and the receptor was covalently bound to the ligand by a photoreaction on the plate. The extent of receptor binding was monitored by the PBAN-R Abs, to which a secondary Ab (goat anti-rabbit) conjugated to HRP was used. The HRP substrate in this format was chemiluminescent. Non-specific binding was determined in the absence of receptor preparation. Both binding assays were applied only to the *H. peltigera* pheromone gland membranes.

2.5.1. Format I

Nunc MaxiSorp 96-wells microplates (Roskilde, Denmark) were coated with 100 μl (10 μg/well) of protein A (affinity-chromatography-purified from *Staphylococcus aureus*, Sigma) in CB, and incubated ON at 4 °C. The wells were then washed three times with PBST, and 100 μl of antiserum (diluted 1:500 in CB containing 0.5% milk powder) were added to the wells and incubated ON at 4 °C. The plates were rinsed with PBST as above and incubated for 1 h at room temperature on an orbital shaker with 100 μl of a receptor-biotinylated ligand complex obtained (in a separate reaction) by incubation of the receptor preparation (at a concentration equivalent to 2 glands/100 μl) with Bpa-PBAN1-33NH_2 ligand (at concentrations ranging from 30 to 1000 pmol/100 μl) in PBS for 75 min, followed by 30 min of 365 nm UV irradiation (at 15 cm from two 15 W UV lamps, at 4 °C). Reaction mixtures prepared in the absence of the biotinylated ligand served to determine non-specific binding. At the end of the 1-h incubation on the microplates, unbound material was washed away with PBST as above, and 100 μl of affinity purified avidin conjugated to HRP (Sigma), diluted 1:400,000 in PBST were added to the plates. After 1 h the plates were rinsed with PBST, and 100 μl of 1-Step Ultra TMB-ELISA Substrate (Pierce, Rockford, IL) were added to each well. The reaction was stopped after 20–30 min by the addition of 50 μl of 4 M sulfuric acid and the absorbance at 450 nm was monitored with an ELISA reader as indicated above.

2.5.2. Format II

White MaxiSorp flat-bottom microplates (NUNC, Roskilde, Denmark) were coated with 100 μl (1 μg) of avidin in CB, and incubated ON at 4 °C. The wells were washed three times with PBST, and 100 μl of biotinylated ligand Bpa-PBAN1-33NH_2, diluted in PBS at concentrations ranging from 10 to 100 pmol/100 μl, were added to the wells, and the plates were incubated for 6 h at room temperature. The wells were washed again with PBST, and 150 μl of 5% normal goat serum (Sigma) diluted in PBS were added, to block non-specific binding sites. The plates were kept ON at 4 °C, and washed again with PBST. Next, 100 μl of receptor preparation in PBS, at a range of concentrations equivalent to 1–3 glands/100 μl were added to the wells, and the plates were incubated for 90 min at room temperature on an orbital shaker. Wells without receptor preparation served to determine non-specific binding. The plates were then UV irradiated at 365 nm as above, washed with PBST, and 100 μl of PBAN-R antiserum diluted 1:4000 in PBS was added to the plates, which were then incubated for 1 h at room temperature and rinsed with PBST, after which 100 μl of goat anti-rabbit conjugated to HRP, diluted 1:20,000 in PBST, were added for 1 h as above. At the end of the incubation, the microplates were rinsed with PBST, and 100 μl of SuperSignal ELISA pico chemiluminescent substrate (Pierce, Rockford, IL) were added. The reaction was monitored for 10–20 min at 1 min intervals with a Lucy 2 chemiluminescent ELISA reader (Anthos, Eugendorf, Austria).

2.6. In vivo bioassays

These assays served to determine whether the antiserum could inhibit *in vivo* binding of PBAN1-33NH_2 to its receptor, and thereby inhibit formation of PBAN-induced cuticular melanization in *S. littoralis* larvae, or pheromone production in *H. peltigera* adult females elicited either by synthetic peptides or by the native endogenous elicitors.

2.6.1. Melanotropic bioassay

2.6.1.1. Synthetic peptide experiments. The bioassay was performed as described previously (Ben-Aziz et al., 2005) using *S. littoralis* larvae that had been reared as described above. Each experiment involved analysis of the intensity of the melanized area in ligated larvae injected with buffer (50 mM HEPES buffer, pH 7.6), PI serum or PBAN-R antiserum (10 μl undiluted) 1 h prior to the injection of 5 pmol of PBAN1-33NH_2 (10 μl in 50 mM HEPES buffer, pH 7.6). The cuticular melanization was quantified as the ratio between the optical density and the scanned cuticular area (in millimeters), and was compared between control (injected with buffer or PI serum) and experimental (injected with antiserum) animals. Experiments were performed with a minimum of nine larvae per treatment.

2.6.1.2. Native endogenous peptide experiments. S. littoralis larvae were injected at head-capsule slippage with 10 μl of antiserum, PI or HEPES buffer. Untreated larvae served as controls. Cuticular melanization was quantified as described above.

2.6.2. Pheromonotropic bioassay

2.6.2.1. Synthetic peptide experiments. The pheromonotropic bioassay was performed with *H. peltigera* as described previously. Briefly, 3.5–4.5-day-old females at the 2nd–4th hour of photophase were injected with 1 or 10 μl of undiluted PBAN-R antiserum, PI serum or buffer at 1, 2, 3, 6 or 24 h prior to injection of 10 μl synthetic PBAN1-33NH_2 (at a dose ranging from 0.3 to 1 pmol), or PT, LPK, MT or ArgPBAN28-33NH_2 (at a dose of 1, 30, 10 or 100 pmol in PBS, respectively), made up in 0.1 M sodium phosphate buffer, pH 7.4. Females injected with 0.1 M phosphate buffer served as controls, to determine the basal pheromone biosynthesis rate at photophase. All experiments were performed with 8–10 females per treatment. At the end of the experiments pheromone glands of injected females were excised and sex pheromone was extracted and quantified by capillary gas chromatography as described previously. The pheromone content in control females did not exceed 10 ng/gland.

2.6.2.2. Native endogenous peptide experiments. Female *H. peltigera* moths were injected at −1 h (i.e., 1 h before the onset of scotophase) with 2, 7.5 or 10 μl of undiluted PBAN-R antiserum, PI serum or buffer, and the pheromone content was excised at the 5th, 6th or 7th hour of scotophase (when pheromone content is high) or after 31 h (i.e., at the 6th hour of the next scotophase). Females injected with buffer served as negative controls, and the pheromone contents in untreated females at the indicated times of scotophase served to determine the maximal pheromone production of the moths. Glands were extracted and checked for pheromone content as above.

2.7. Statistical analysis

The results of the pheromonotropic and melanotropic assays were subjected to one-way ANOVA. The significance of differences among means was evaluated with the Tukey–Kramer HSD (honestly significant difference) test at $P < 0.05$.

3. Results

3.1. Characterization of the PBAN-R antiserum

First, the antiserum was tested for its ability to recognize the hapten against which it has been generated. For this purpose, an ELISA was established and the cross-reactivity of the antiserum was tested by comparing the ability of the hapten against which the antiserum was generated (PBAN-R-21-35) to compete with a biotinylated hapten adsorbed onto the microplate via avidin in binding to the antiserum. The results (Fig. 2) revealed that the antiserum did recognize the hapten within a range of 0.003–300 ng with an I_{50} of 1 ng. The antiserum did not cross-react with the full-length PBAN molecule (PBAN1-33NH_2) or with a C-terminally derived PBAN peptide (PBAN28-33NH_2, which constitutes the active site of the PK/PBAN family), in amounts up to 300 ng (Fig. 2). The antiserum also did not cross-react with the biotinylated PBAN molecules (Bpa-PBAN1-33NH_2 and Bpa-ArgPBAN28-33NH_2, data not shown).

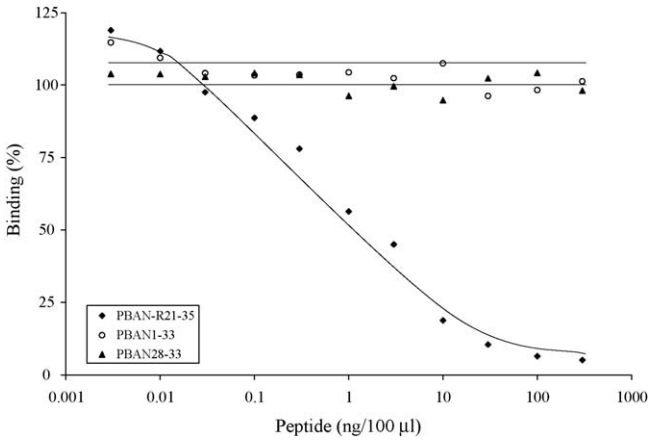

Fig. 2. Cross-reactivity of PBAN-R antiserum with the peptide against which it had been generated (PBAN-R-21-35), and two PBAN molecules: the full-length PBAN (PBAN1-33NH_2) and a PBAN C-terminal-derived peptide (PBAN28-33NH_2).

The ability of the antiserum to recognize the PBAN-R from pheromone glands was tested by dot blot analysis. A membraneous preparation (containing the PBAN-R, Altstein et al., 2001) was obtained from of *H. peltigera* female pheromone glands. The membranes were adsorbed onto a PVDF membrane filter and the ability of the antiserum to specifically detect the receptor was tested at various antiserum concentrations. The extent of binding was determined with a secondary antibody raised in goats against rabbit IgG conjugated to a reporting enzyme HRP. The data in Fig. 3A clearly indicate the ability of the antiserum to recognize the PBAN-R of the pheromone gland receptor preparation. The response was found to be dose-dependent with respect to the primary and secondary Abs. No binding of the PBAN-R antiserum could be detected when ovalbumin (at an amount equivalent to the protein content of the PBAN-R preparation) was adsorbed onto the PVDF filter, instead of the PBAN-R preparation. A weak reaction was detected at concentrations of 1:500 and 1:25,000 of primary and secondary Abs, respectively, most likely caused by non-specific adsorption of the primary or the secondary Abs (or both) to the protein or the PVDF membrane. At all other concentration non-specific binding was negligible. Preimmune serum did not generate any significant signal, indicating, together with the ovalbumin data, that the binding of the PBAN-R to the gland preparation was specific. The reaction also was dose-dependent with respect to the number of glands used for binding (Fig. 3B), and the antiserum recognized the PBAN-R in pheromone gland receptor preparations of two other female moths – *H. armigera* and *S. littoralis* – in a specific and dose-dependent manner (Fig. 3C).

3.2. Employment of the antiserum for activity inhibition in adult moths and larvae

The ability of the antiserum to inhibit receptor activation (i.e., sex pheromone production and melanin formation) *in vivo* was evaluated in adult female *H. peltigera* moths and in *S. littoralis*

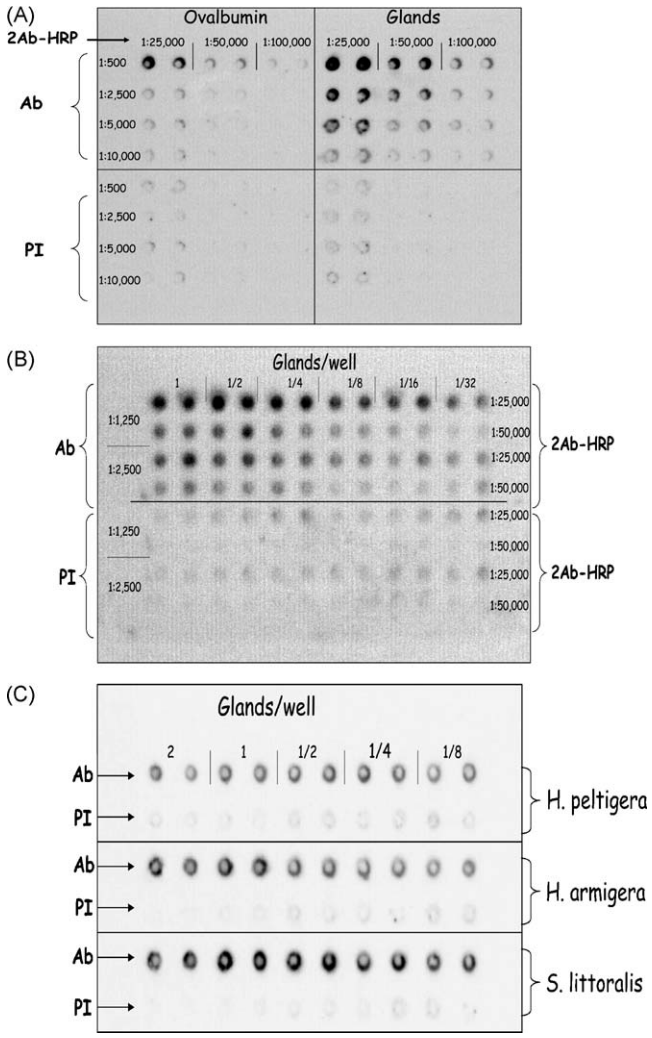

Fig. 3. Dot blot analysis. (A) Specific binding of PBAN-R antiserum to *H. peltigera* pheromone gland receptor preparations at various PI serum, primary Ab (Ab) and secondary Ab (2Ab-HRP) dilutions. The amount of protein (ovalbumin) and gland preparation was 4 μg, equivalent to the amount of protein in one pheromone gland. Dilutions of PI serum and primary Abs are indicated on the left. Amounts of secondary Ab (goat anti-rabbit) conjugated to HRP is indicated on top. (B) Dose–response analysis of PBAN-R antiserum with various amounts of gland equivalents at various dilutions of PI serum, and primary and secondary Abs. (C) Dose–response analysis of PBAN-R antiserum with various amounts of gland equivalents obtained from various moth species. Ab and PI serum were used at a dilution of 1:2,500 and goat anti-rabbit HRP secondary Ab at a dilution of 1:25,000.

larvae, respectively. We tested the inhibitory potency of the antiserum on the above functions, which had been elicited either by injected synthetic PBAN or by the native mechanism, which involves the endogenous peptides. Injection of PBAN-R antiserum (10 μl undiluted) into *S. littoralis* larvae, at the head-capsule slippage stage, 1 h prior to the injection of an elicitor (5 pmol PBAN1-33NH_2) resulted in full inhibition of cuticular melanin formation (Fig. 4). The inhibition by the antiserum was specific; injection of the PI serum under similar experimental conditions did not have any inhibitory affect, and the degree of melanization in the latter case did not differ significantly from that following injection of buffer. The antiserum also inhibited cuticular melanization elicited by the endogenous mechanism, and injection of 10 μl antiserum into larvae at head-capsule slippage resulted in a significantly lower degree of melanization (33%) than that obtained in insects injected with PI serum or buffer.

Injection of 1 or 10 μl of the undiluted antiserum into adult *H. peltigera* females at photophase, 1, 2, 3, 6 or 24 h prior to injection

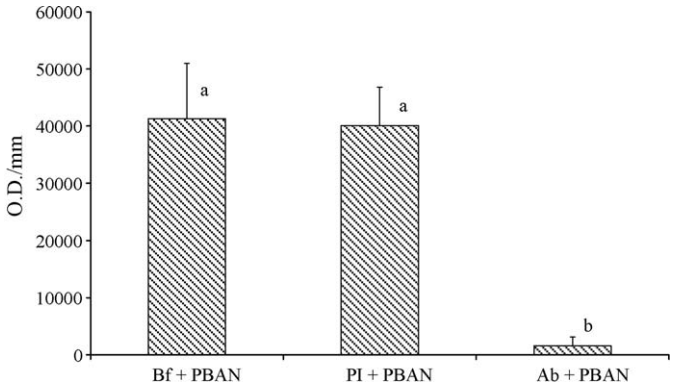

Fig. 4. Effects of preimmune (PI) serum and PBAN-R antiserum (Ab) on the ability of synthetic PBAN1-33NH_2 to induce cuticular melanization in *S. littoralis* larvae. Larvae were injected with 10 μl of 50 mM HEPES buffer, pH 7.6 (Bf), PI serum or undiluted PBAN-R antiserum (Ab), ligated and left for 1 h at room temperature. The first injection was followed by injection of 5 pmol PBAN1-33NH_2 in the same buffer and a second ligation. Bars with different letters indicate an extent of melanization that differs significantly (at $P < 0.05$) from that induced by PBAN1-33NH_2 (at 5 pmol).

of diverse doses of a variety of elicitors—PBAN1-33NH_2 at 0.3, 0.5 or 1 pmol, or PT, LPK, MT or the C-terminally derived analog Arg-PBAN28-33NH_2 at 1, 30, 10 or 100 pmol, respectively, did not inhibit sex pheromone production, and the level of pheromone synthesized under all tested conditions was similar to that obtained following injection of PI serum or buffer.

Examination of the ability of the antiserum to inhibit pheromone production elicited by the natural endogenous mechanism showed similar results. Injection of 2, 7.5 or 10 μl of undiluted PBAN-R antiserum, followed by analysis of the pheromone content at the 5th, 6th or 7th hour of scotophase, when pheromone content is high, or after 31 h, i.e., at the 6th hour of the subsequent scotophase, revealed pheromone levels which were undistinguishable from those elicited by injection of PI serum or buffer.

3.3. Employment of the antiserum for the development of a receptor binding assay

Two assay formats were developed, both of which used photo-affinity biotinylated ligands. In both formats binding was found to be specific and dose-dependent with respect to the amount of ligand, within the ranges of 30–1000 and 10–100 pmol in formats I and II, respectively (Figs. 5 and 6). Binding was dose-dependent

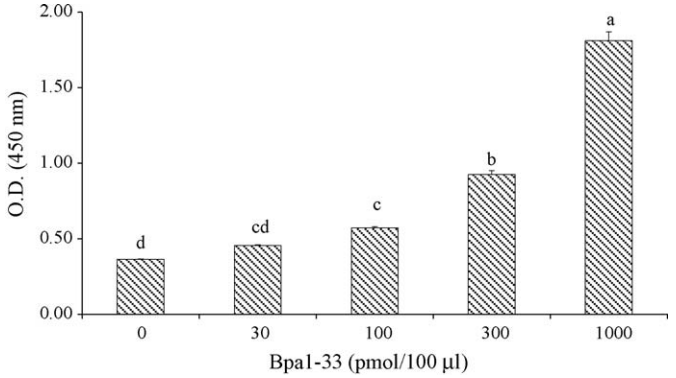

Fig. 5. Dose–response of ligand (biotinylated photo-affinity PBAN, Bpa1-33) binding to *H. peltigera* PBAN-R adsorbed to the microplate *via* PBAN-R antiserum. The reaction was carried out by using the format 1 binding assay with PBAN-R obtained from two gland equivalents per point. Each point represents the mean ± SEM ($n = 2$). Statistical analysis compared binding at the different doses. Bars with different letters differ from each other at $P < 0.05$.

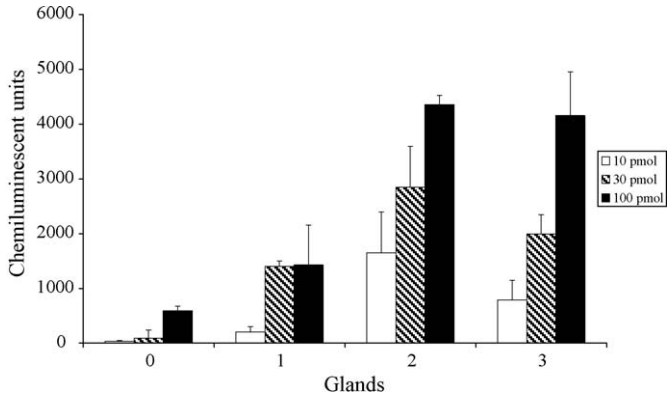

Fig. 6. Dose–response curve of ligand binding to various amounts of PBAN-R preparations. Glands were obtained from *H. peltigera* pheromone gland and covalently adsorbed to the microplate *via* a photo-affinity biotinylated ligand (Bpa-PBAN1-33NH_2). The reaction was carried out by using the format 2 binding assay. Each point represents the mean ± SEM (n = 2).

with respect to the amount of gland equivalents present in the reaction (Fig. 6).

4. Discussion

In this study, we generated and characterized a highly specific anti-PBAN antiserum, and used it to characterize the PK/PBAN-R(s) that mediate sex pheromone biosynthesis in adult female *H. peltigera* moths and cuticular melanization in *S. littoralis* larvae. We also used it to develop a 96-well microplate receptor binding assay. The antiserum was generated against a synthetic peptide, derived from the nucleotide sequence of the PBAN-R of the cloned *H. zea* pheromone gland; a sequence that corresponds to amino acids 21-35 of the N-terminus and is considered part of the binding site of peptides to GPCRs. The sequence was chosen in light of the high similarity the peptide displayed with various receptors of the PK/PBAN family cloned from the pheromone gland of adult females and larvae: *Heliothis virescens* (Kim et al., 2008), *S. littoralis* (Zheng et al., 2007), *H. armigera* and *S. exigua* (GenBank accession no. AY792036 and EU365878.2, respectively) and the very low sequence similarity it displayed with sequences of all other GPCRs, as indicated by BLAST analyses. This was important, in order to increase the probability of generating a highly specific antiserum for the PBAN-R family. The resulting antiserum recognized the hapten against which it had been generated and did not cross-react with PBAN or with peptides derived from its sequence (Fig. 2).

The antiserum recognized, in a specific and dose-dependent manner, the PBAN-R present in membrane preparations of *H. peltigera*, *H. armigera* and *S. littoralis* pheromone glands (Fig. 3). Also, in a histochemical study, the antiserum stained the pheromone gland epithelial cells of *H. peltigera* with high specificity (data not shown). Although the pheromone gland receptors of *H. peltigera* and *S. littoralis* have not been cloned, and their sequences have not yet been determined, it is reasonable to assume, in light of the above data, that the N-terminal parts of these receptors (between amino acids 21-35) are highly conserved.

In addition to recognizing the *H. peltigera* pheromone gland PK/PBAN-R of adult female moths *in vitro*, the antiserum also recognized the native *S. littoralis* larval receptor *in vivo*. Injection of the PBAN-R antiserum into *S. littoralis* larvae at the head-capsule slippage stage resulted in a full inhibition of cuticular melanin formation, which was induced by synthetic PBAN1-33NH_2 (Fig. 4), which indicates the possibility that it competes or interferes (by steric hindrance) with the docking of the elicitor (i.e., ligand), and that the fragment between amino acids 21-35 (against which the Ab was raised) plays a major role in the binding of the ligand to its receptor, and either constitutes the active site or is in close proximity to it. The antiserum also inhibited melanization elicited by the endogenous native peptide, although to a lesser extent (33%). The inhibition by the antiserum was specific, and injection of PI serum under similar experimental conditions did not have any inhibitory affect, and the degree of melanization in the latter case did not differ from that elicited by buffer injection. Currently, it is not clear why the antiserum can fully inhibit cuticular melanization elicited by the synthetic peptide but can only partially inhibit melanization elicited by the native peptide. One possible explanation is that the endogenous elicitor differs from the synthetic peptide that was used to elicit melanization in the above experiments, i.e., that it is another member of the PK/PBAN family, and binds to the receptor in a different region, which is not within the fragment between amino acids 21-35 and to which the Ab does not bind. Alternatively, it is possible that the cuticular melanization is activated by different receptor subtypes and that the endogenous elicitor binds to a subtype that is not recognized by the Ab.

Interestingly, the antiserum did not recognize the *H. peltigera* pheromone gland receptor *in vivo*, despite the fact that it had been generated against a peptide derived from the sequence of the pheromone gland receptor, and despite its ability to recognize the receptor present in receptor preparations in dot blot experiments. Injection of the antiserum into adult *H. peltigera* females did not affect the ability of the females to synthesize pheromone elicited either by an injected synthetic peptide or by the endogenous native elicitor. These findings can also be explained as above (by involvement of different elicitors in both functions), however, since PBAN was found in many adult female moths and larvae (Altstein and Hariton, in press), the second of the above explanations, namely, existence of receptor subtypes is more likely than the assumption that pheromone biosynthesis is evoked by an elicitor other than PBAN. The ability of the antiserum to recognize the pheromone gland receptor in the dot blot experiments may have arisen from modifications of its structure in the course of the membrane preparation in a manner that enabled its recognition by the antiserum.

The possible existence of receptor subtypes among the PK/PBAN family was also suggested in our previous studies, which were based on receptor cloning and selective bioactivity of various PK/PBAN agonists and antagonists. In pursuing this investigation we recently cloned a PK/PBAN receptor that mediated cuticular melanization, from *S. littoralis* larvae, and evaluated the structure of its gene. Although the receptor exhibited high sequence similarity with the pheromone gland receptors of *H. zea*, *B. mori* and *H. virescens*, differences were noticed in parts of the N-terminal region, the second outer loop, the third inner loop and in the C-terminal inner part. Currently, the structural mechanisms of the interactions between ligands and GPCRs are still not clear, but the data on GPCRs that have been accumulated so far suggest that the extracellular N-terminus/loops are responsible for the ligand docking/interaction (Leff, 1995). It is thus possible that the major difference observed in the N-terminal region of the PBAN-Rs may account for optimal conspecific ligand/receptor docking and interaction, which results in different ligands, e.g., elicitors, exhibiting differing patterns of binding to the pheromone gland and larval receptors. The TM helices that are highly conserved between different PBAN-Rs might form a precise ligand interaction pocket, which is required to enable the FXPRLa motif-induced receptor conformational change and receptor activation.

The possible existence of PBAN-R subtypes was also suggested by us in light of our previous studies with various PK/PBAN analogs: conformationally constrained agonists and antagonists (Altstein and Hariton, in press); two *trans*Pro mimetic analogs Ac-YF[Jo]RLa (PPK-Jo: PBAN Pyrokinin—Jones moiety) (Nachman et al.,

2009a), and Ac-YF[Etz]RLa (PK-Etz, Nachman et al., 2009d); and the β^2 and β^3 amino acid-substituted peptides, Ac-YFT[β^3P]RLa (PK-βA-1), Ac-Y[β^2homoF]TPRLa (PK-βA-2), Ac-Y[β^3F]TPRLa (PK-βA-3), and Ac-[β^3F]FT[β^3P]RLa (PK-βA-4) (Nachman et al., 2009b). Backbone cyclic (BBC) peptides, which display a much higher selectivity toward receptors because of the restriction of their conformational space, PPK-Jo and Ac-YF[Etz]RLa which lock the peptide in a *trans*Pro conformation by the introduction of a dihydroimidazoline or an (*E*)-alkene moiety, and the β^2 and β^3 peptides all differed in their ability to stimulate or inhibit various functions, i.e., sex pheromone production in adult moths, melanization in moth larvae, pupariation in flesh flies, and hindgut contraction in cockroaches. For example: BBC-25 inhibited sex pheromone biosynthesis in *H. peltigera* females that was elicited by PBAN but not by PT, LPK or MT; and BBC-22 inhibited only PBAN-elicited pheromone biosynthesis but not any other function mediated by any of the tested PK/PBAN elicitors. Clear elucidation regarding the existence of PBAN receptor subtypes will depend on further evaluation based on cloning information, SAR analysis by means of 3D modeling of cloned receptor(s), and evaluation of their interactions with the various ligands and inhibitors, or the use of Abs directed against different regions of the receptor such as those developed in the course of the present study.

Abs are very powerful tools for characterization of GPCRs and other receptors, and they can provide answers to many of the questions raised above, regarding the modes of action of a multifunctional Np family such as the PK/PBAN family. These advantages spring from their high specificity and the relative ease of tailoring them according to need. Abs have been used for many applications including, purification, localization and characterization of mammalian GPCRs. Antisera targeted against defined regions of various receptors have proved useful: in detecting functional groups involved in ligand/receptor interaction (in human β^2 adrenergic receptors (Mijares et al., 1996); in mapping the binding sites and characterizing receptor subtypes (neuropeptide Y (Eckard et al., 1999) and D_2 dopamine receptor (David and Fuchs, 1991)); in elucidating the role of phosphorylation in desensitization (δ-opioid receptor (Hasbi et al., 1998); in determining the distribution of receptors in the plasma membrane (e.g., bradykinin B_2 receptor, Haasemann et al., 1998); in internalization (muscarinic cholinergic receptor, Tolbert and Lameh, 1998) and in characterization of G-protein assembly. Anti-receptor Abs were also used in immunocytochemical studies to localize insects GPCRs such as the leucokinin, proctolin and diuretic hormone receptors of *Drosophila melanogaster* (Radford et al., 2002; Johnson et al., 2003, 2005). The Abs described in the present paper are, to the best of our knowledge, the first to be implemented for *in vivo* studies in insects, and their use in characterizing the PK/PBAN receptors may, therefore, further provide important information on the mode of action of this Np family.

The broad physiological relevance of PBAN and other peptide of the family, and the growing number of indications of the multiplicity of its receptors, or of the existence of receptor subtypes, account for the increasing interest in the PK/PBAN family as a new target in the search for insect control agents based on Np antagonists. In the past few years we carried out an extensive study of the design of receptor selective PK/PBAN antagonists; a study that led to the generation of several highly potent, conformationally constrained, selective, metabolically stable and bioavailable compounds (for a detailed review see Altstein and Hariton, in press). These compounds were the first PBAN antagonists to be discovered, and they formed the basis for the development of a second generation of antagonists that can serve as insecticide prototypes, i.e., cost-effective, small molecules.

Conversion of the peptide antagonist into a non-peptide small molecule (SM), requires identification of the biophores that are essential for the antagonistic activity, and their incorporation into novel, scaffold SM libraries that can be further screened for the desired compounds by means of a high-throughput screening assay (HTSA). As a step toward this goal the antiserum developed in the course of the present study was used to develop an HTSA platform. Two microplate assay formats were developed, both of which used photo-affinity biotinylated ligands. In both formats binding was found to be specific and dose-dependent with respect to the amount of ligand (Figs. 5 and 6) and with respect to the amount of receptor preparation present in the reaction (Fig. 6). Many HTSA based on GPCRs have been developed so far, based on a large variety of mammalian and other receptors intended for drug discovery, for discovery of lead compounds or for target validation (Eglen, 2005). Only one insect HTA was reported, so far, for screening ecdysteroid agonist and antagonists based on the insect steroid hormone ecdysone-response element (Swevers et al., 2004), and, to the best of our knowledge, no HTSAs were yet developed based on insect GPCRs. The Abs and the microplate assay that were developed in the present study, in combination with a *S. littoralis* PK/PBAN receptor that has recently been cloned (Zheng et al., 2007), and expressed in insect Sf-9 cells, will enable development of an insect GPCR-based HTSA for discovery of new control agents based on Np antagonists of this family of peptides, in natural, chemical and molecular combinatorial libraries.

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References

Altstein, M., Ben-Aziz, O., Bhargava, K., Li, Q.J., Martins-Green, M., 2003. Histochemical localization of the PBAN receptor in the pheromone gland of *Heliothis peltigera*. Peptides 24, 1335–1347.

Altstein, M., Ben-Aziz, O., Daniel, S., Schefler, I., Zeltser, I., Gilon, C., 1999. Backbone cyclic peptide antagonists, derived from the insect pheromone biosynthesis activating neuropeptide, inhibit sex pheromone biosynthesis in moths. J. Biol. Chem. 274, 17573–17579.

Altstein, M., Ben-Aziz, O., Daniel, S., Zeltser, I., Gilon, C., 2001. Pyrokinin/PBAN radioreceptor assay: development and application for the characterization of a putative receptor from the pheromone gland of *Heliothis peltigera*. Peptides 22, 1379–1389.

Altstein, M., Ben-Aziz, O., Zeltser, I., Bhargava, K., Davidovitch, M., Strey, A., Pryor, N., Nachman, R.J., 2007. Inhibition of PK/PBAN-mediated functions in insects: discovery of selective and non-selective inhibitors. Peptides 28, 574–584.

Altstein, M., Dunkelblum, E., Gabay, T., Ben Aziz, O., Schafler, I., Gazit, Y., 1995. PBAN-Induced sex-pheromone biosynthesis in *Heliothis peltigera*: structure, dose, and time-dependent analysis. Arch. Insect Biochem. Physiol. 30, 307–319.

Altstein, M., Gazit, Y., Ben Aziz, O., Gabay, T., Marcus, R., Vogel, Z., Barg, J., 1996. Induction of cuticular melanization in *Spodoptera littoralis* larvae by PBAN/MRCH: development of a quantitative bioassay and structure function analysis. Arch. Insect Biochem. Physiol. 31, 355–370.

Altstein, M., Hariton, A., in press. Rational design of insect control agents: the PK/PBAN family as a study case. In: Biorational Control of Arthropod Pests: Application and Resistance Management. In: Ishaaya, I., Horovitz, R, (Eds.), Springer.

Ben-Aziz, O., Zeltser, I., Altstein, M., 2005. PBAN selective antagonists: inhibition of PBAN induced cuticular melanization and sex pheromone biosynthesis in moths. J. Insect Physiol. 51, 305–314.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Cazzamali, G., Torp, M., Hauser, F., Williamson, M., Grimmelikhuijzen, C.J.P., 2005. The Drosophila gene CG9918 codes for a pyrokinin-1 receptor. Biochem. Biophys. Res. Commun. 335, 14–19.

Choi, M.Y., Fuerst, E.J., Rafaeli, A., Jurenka, R., 2003. Identification of a G protein-coupled receptor for pheromone biosynthesis activating neuropeptide from pheromone glands of the moth *Helicoverpa zea*. Proc. Natl. Acad. Sci. U.S.A. 100, 9721–9726.

David, C., Fuchs, S., 1991. Antipeptide antibodies localize N-(4-azido-3-[125I] iodophenethyl)spiperone binding to the carboxyl-terminal portion of the D2 dopamine receptor. Mol. Pharmacol. 40, 712–716.

Dunkelblum, E., Kehat, M., 1989. Female sex-pheromone components of *Heliothis peltigera* (Lepidoptera: Noctuidae) chemical-identification from gland extracts and male-response. J. Chem. Ecol. 15, 2233–2245.

Eckard, C.P., Beck-Sickinger, A.G., Wieland, H.A., 1999. Comparison of antibodies directed against receptor segments of NPY-receptors. J. Recept. Signal Transduct. Res. 19, 379–394.

Eglen, M.R., 2005. An overview of high throughput screening at g protein coupled receptors. Front. Drug Des. Discov. 1, 97–111.

Gade, G., 1997. The explosion of structural information on insect neuropeptides. Prog. Chem. Org. Nat. Prod. 71, 1–128.

Gazit, Y., Dunkelblum, E., Benichis, M., Altstein, M., 1990. Effect of synthetic PBAN and derived peptides on sex-pheromone biosynthesis in *Heliothis peltigera* (Lepidoptera, Noctuidae). Insect Biochem. 20, 853–858.

Haasemann, M., Cartaud, J., Muller-Esterl, W., Dunia, I., 1998. Agonist-induced redistribution of bradykinin B2 receptor in caveolae. J. Cell Sci. 111 (Pt 7), 917–928.

Hasbi, A., Polastron, J., Allouche, S., Stanasila, L., Jauzac, P., 1998. Desensitization of the delta-opioid receptor correlates with its phosphorylation in SK-N-BE cells: involvement of a G protein-coupled receptor kinase. J. Neurochem. 70, 2129–2133.

Holman, G.M., Cook, B.J., Nachman, R.J., 1986. Isolation, primary structure and synthesis of a blocked myotropic neuropeptide isolated from the cockroach, *Leucophaea maderae*. Comp. Biochem. Physiol. C-Pharmacol. Toxicol. Endocrinol. 85, 219–224.

Homma, T., Watanabe, K., Tsurumaru, S., Kataoka, H., Imai, K., Kamba, M., Niimi, T., Yamashita, O., Yaginuma, T., 2006. G protein-coupled receptor for diapause hormone, an inducer of Bombyx embryonic diapause. Biochem. Biophys. Res. Commun. 344, 386–393.

Hull, J.J., Ohnishi, A., Moto, K., Kawasaki, Y., Kurata, R., Suzuki, M.G., Matsumoto, S., 2004. Cloning and characterization of the pheromone biosynthesis activating neuropeptide receptor from the silkmoth, Bombyx mori—significance of the carboxyl terminus in receptor internalization. J. Biol. Chem. 279, 51500–51507.

Johnson, E.C., Shafer, O.T., Trigg, J.S., Park, J., Schooley, D.A., Dow, J.A., Tagher, P.H., 2005. A novel diuretic hormone receptor in *Drosophila*: evidence for conservation of CGRP signaling. J. Exp. Biol. 208, 1239–1246.

Johnson, E.C., Garcznski, S.F., Park, D., Crim, J.W., Nässel, D.R., Taghert, P.H., 2003. Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 100, 6198–6203.

Imai, K., Konno, T., Nakazawa, Y., Komiya, T., Isobe, M., Koga, K., Goto, T., Yaginuma, T., Sakakibara, K., Hasegawa, K., Yamashita, O., 1991. Isolation and structure of diapause hormone of the silkworm, *Bombyx mori*. Proc. Jpn. Acad. Ser. B-Phys. Biol. Sci. 67, 98–101.

Kean, L., Cazenave, W., Costes, L., Broderick, K.E., Graham, S., Pollock, V.P., Davies, S.A., Veenstra, J.A., Dow, J.A.T., 2002. Two nitridergic peptides are encoded by the gene capability in *Drosophila melanogaster*. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 282, R1297–R1307.

Kim, Y.J., Nachman, R.J., Aimanova, K., Gill, S., Adams, M.E., 2008. The pheromone biosynthesis activating neuropeptide (PBAN) receptor of Heliothis virescens: identification, functional expression, and structure-activity relationships of ligand analogs. Peptides 29, 268–275.

Leff, P., 1995. The 2-state model of receptor activation. Trends Pharmacol. Sci. 16, 89–97.

Matsumoto, S., Fonagy, A., Kurihara, M., Uchiumi, K., Nagamine, T., Chijimatsu, M., Mitsui, T., 1992. Isolation and primary structure of a novel pheromonotropic neuropeptide structurally related to leucopyrokinin from the armyworm larvae, *Pseudaletia separata*. Biochem. Biophys. Res. Commun. 182, 534–539.

Matsumoto, S., Kitamura, A., Nagasawa, H., Kataoka, H., Orikasa, C., Mitsui, T., Suzuki, A., 1990. Functional diversity of a neurohormone produced by the subesophageal ganglion: molecular identity of melanization and reddish coloration hormone and pheromone biosynthesis activating neuropeptide. J. Insect Physiol. 36, 427–432.

Meng, X.J., Wahlstrom, G., Immonen, T., Kolmer, M., Tirronen, M., Predel, R., Kalkkinen, N., Heino, T.I., Sariola, H., Roos, C., 2002. The Drosophila hugin gene codes for myostimulatory and ecdysis-modifying neuropeptides. Mech. Dev. 117, 5–13.

Mijares, A., Lebesgue, D., Argibay, J., Hoebeke, J., 1996. Anti-peptide antibodies sensitive to the 'active' state of the beta2-adrenergic receptor. FEBS Lett. 399, 188–191.

Nachman, R.J., Ben-Aziz, O., Davidovitch, M., Kaczmarek, K., Zabrocki, J., Williams, H., Strey, A., Altstein, M., 2009a. A novel dihydroimidazoline, trans-Pro mimetic analog is a selective PK/PBAN agonist. Front. Biosci., in press.

Nachman, R.J., Ben-Aziz, O., Davidovitch, M., Zubrzak, P., Isaac, E., Strey, A., Reyes-Rangel, G., Juaristi, E., Williams, H.J., Altstein, M., 2009b. Biostable beta-amino acid PK/PBAN analogs: agonist and antagonist properties. Peptides 30, 609–615.

Nachman, R.J., Wang, X.J., Etzkorn, F.A., Ben Aziz, O., Davidovitch, M., Kaczmarek, K., Zabrocki, J., Strey, A., Pryor, N., Altstein, M., 2009d. Evaluation of a PK/PBAN analog with an (E)-alkene, trans-Pro isostere identifies the Pro orientation for activity in four diverse PK/PBAN bioassays. Peptides, in press.

Nachman, R.J., Holman, G.M., 1991. Myotropic insect neuropeptide families from the cockroach *Leucophaea maderae*—structure–activity relationships. ACS Symp. Ser. 453, 194–214.

Nachman, R.J., Holman, G.M., Cook, B.J., 1986. Active fragments and analogs of the insect neuropeptide leucopyrokinin: structure–function studies. Biochem. Biophys. Res. Commun. 137, 936–942.

Nachman, R.J., Teal, P.E.A., Ben-Aziz, O., Davidovitch, M., Zubrzak, P., Altstein, M., 2009c. An amphiphilic, PK/PBAN analog is a selective pheromonotropic antagonist that penetrates the cuticle of a heliothine insect. Peptides 30, 616–621.

Nachman, R.J., Zdarek, J., Holman, G.M., Hayes, T.K., 1997. Pupariation acceleration in fleshfly (*Sarcophaga bullata*) larvae by the pyrokinin/PBAN neuropeptide family—structure–activity relationships. Ann. N. Y. Acad. Sci. 814, 73–79.

Olsen, S.S., Cazzamali, G., Williamson, M., Grimmelikhuijzen, C.J.P., Hauser, F., 2007. Identification of one capa and two pyrokinin receptors from the malaria mosquito Anopheles gambiae. Biochem. Biophys. Res. Commun. 362, 245–251.

Predel, R., Eckert, M., 2000. Tagma-specific distribution of FXPRLamides in the nervous system of the American cockroach. J. Comp. Neurol. 419, 352–363.

Predel, R., Kellner, R., Nachman, R.J., Holman, G.M., Rapus, J., Gade, G., 1999. Differential distribution of pyrokinin-isoforms in cerebral and abdominal neurohemal organs of the American cockroach. Insect Biochem. Mol. Biol. 29, 139–144.

Radford, J.C., Davies, S.A., Dow, J.A.T., 2002. Systematic G-protein-coupled receptor analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. J. Biol. Chem. 277, 38810–38817.

Raina, A.K., Jaffe, H., Kempe, T.G., Keim, P., Blacher, R.W., Fales, H.M., Riley, C.T., Klun, J.A., Ridgway, R.L., Hayes, D.K., 1989. Identification of a neuropeptide hormone that regulates sex-pheromone production in female moths. Science 244, 796–798.

Raina, A.K., Klun, J.A., 1984. Brain factor control of sex-pheromone production in the female corn-earworm moth. Science 225, 531–533.

Rosenkilde, C., Cazzamali, G., Williamson, M., Hauser, F., Sondergaard, L., DeLotto, R., Grimmelikhuijzen, C.J.P., 2003. Molecular cloning, functional expression, and gene silencing of two Drosophila receptors for the Drosophila neuropeptide pyrokinin-2. Biochem. Biophys. Res. Commun. 309, 485–494.

Schoofs, L., Holman, G.M., Hayes, T.K., Nachman, R.J., Deloof, A., 1991. Isolation, primary structure, and synthesis of Locustapyrokinin—a myotropic peptide of *Locusta migratoria*. Gen. Comp. Endocrinol. 81, 97–104.

Schoofs, L., Vandenbroeck, J., Deloof, A., 1993. The myotropic peptides of *Locusta migratoria*: structures, distribution, functions and receptors. Insect Biochem. Mol. Biol. 23, 859–881.

Swevers, L., Kravariti, L., Ciolfi, S., Xenou-Kokoletsi, M., Ragoussis, N., Smagghe, G., Nakagawa, Y., Mazomenos, B., Iatrou, K., 2004. A cell-based high-throughput screening system for detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic compounds. FASEB J. 18, 134–136.

Tolbert, L.M., Lameh, J., 1998. Antibody to epitope tag induces internalization of human muscarinic subtype 1 receptor. J. Neurochem. 70, 113–119.

Veelaert, D., Schoofs, L., Verhaert, P., De Loof, A., 1997. Identification of two novel peptides from the central nervous system of the desert locust, *Schistocerca gregaria*. Biochem. Biophys. Res. Commun. 241, 530–534.

Zeltser, I., Ben-Aziz, O., Schefler, I., Bhargava, K., Altstein, M., Gilon, C., 2001. Insect neuropeptide antagonist. Part II. Synthesis and biological activity of backbone cyclic and precyclic PBAN antagonists. J. Pept. Res. 58, 275–284.

Zeltser, I., Gilon, C., Ben-Aziz, O., Schefler, I., Altstein, M., 2000. Discovery of a linear lead antagonist to the insect pheromone biosynthesis activating neuropeptide (PBAN). Peptides 21, 1457–1465.

Zheng, L., Lytle, C., Njauw, C.-N., Altstein, M., Martins-Green, M., 2007. Cloning and characterization of the pheromone biosynthesis activating neuropeptide receptor gene in *Spodoptera littoralis* larvae. Gene 393, 20–30.