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PEPTIDES

Peptides 22 (2001) 1379–1389

Pyrokinin/PBAN radio-receptor assay: development and application for the characterization of a putative receptor from the pheromone gland of *Heliothis peltigera*☆

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Received 2 March 2001; accepted 18 April 2001

Abstract

A radio-receptor assay (RRA) for the insect pyrokinin/PBAN family has been developed. The development involved examination of the ligand (^{3}H-tyrosyl-PBAN28–33NH$_2$)-receptor interaction under various incubation conditions and variations on sex pheromone gland membrane preparation. Application of the RRA for a partial characterization of the putative pyrokinin/PBAN receptor in the pheromone gland of *H. peltigera* revealed age-dependence of its expression. Pharmacological characterization revealed a high correlation between the binding-affinity to the receptor of various PBAN-derived peptides and their in vivo pheromonotropic bioactivity, and shed light on the interaction of backbone cyclic and linear ([Arg^{27},D-Phe^{30}]PBAN28–33NH$_2$) PBAN antagonists with the receptor.

Keywords: PBAN receptor; PBAN; pheromone gland; *Heliothis peltigera;* Insect neuropeptide; moths; radio receptor assay

1. Introduction

Pheromone biosynthesis activating neuropeptide (PBAN) is an important neuropeptide that mediates some of the key functions in insects. It was first reported as the neuropeptide that regulates sex pheromone production in female moths [49], and its amino acid sequence was determined in 1989 for *Helicoverpa zea* (Hez-PBAN; PBAN1–33NH$_2$) [50]. Since then, six other PBAN molecules have been isolated from five additional moth species [12,17,25, 28,29,34] and the c-DNA and genes of PBAN have been cloned [12,14,17,18,25–27,33]. The PBAN family consists of C-terminally amidated peptides of 33–34 amino acid residues. All peptides share a high degree of homology and an identical C-terminal pentapeptide sequence (Phe-Ser-Pro-Arg-Leu-NH$_2$) that also constitutes the active core required for their biological activity [1–3,5,30,42,47,48]. The common C-terminal sequence (Phe-Xxx-Pro-Arg-Leu-NH$_2$; Xxx = Ser, Gly, Thr, Val) is also shared by additional insect neuropeptides, all of which have been designated the pyrokinin/PBAN family, comprising: melanization and reddish coloration hormone (MRCH) [35], pyrokinins and myotropins [40,56], pheromonotropin [36] and diapause hormone [24].

To date, the presence of members of the pyrokinin/PBAN family has been demonstrated in a variety of moths as well as in non-lepidopteran species, and their mode of action has been studied in many laboratories [21,46,57]. These studies have revealed that in addition to their ability to stimulate sex pheromone biosynthesis in moths, members of this family have been found to control melanization and reddish coloration in moth larvae [4,35], contraction of the locust oviduct [55], myotropic activity of the cockroach and locust hindgut [39,55], egg diapause in the silkworm [24] and acceleration of pupariation in fleshfly larvae [41]. The major role of PBAN and the pyrokinin/PBAN family in the physiology of moths and other insects, and their functional diversity stimulated us to study the receptor(s) associated with this family of neuropeptides.

Characterization of a neurohormone receptor usually requires the development of a receptor-binding assay that may then be applied to biological and pharmacological analyses.

☆ Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. No 505/01, 2001 series.

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0196-9781/01/$ – see front matter
PII: S0196-9781(01)00479-X

Such information may lead to a better understanding of the pharmacological properties of the receptor; disclose the existence of multiple receptor families; evaluate its distribution in different organs and its presence in different species, etc. Once such an assay is established, it may serve as a basis for the development of a high-throughput screening assay (HTSA) that can be applied to combinatorial and conformational peptidic and non-peptidic libraries in the search for agonists and antagonists (which themselves offer great potential for a further understanding of the role of a given peptide in the physiology of the insect). Screening of combinatorial and conformational peptidic and non-peptidic libraries may lead to the discovery of selective and non-selective agonists and antagonists that can be further used for the characterization and localization of new receptors.

Currently, several receptors of insect peptide hormones have been characterized. The proctolin binding sites from the locust hindgut and the cockroach fore-and hindguts have been characterized in a membranous preparation [37,45]. The adipokinetic hormone receptor from the fat body has been studied in *Manduca Sexta* [64] and in the face fly, *Musca autumnalis* [38]. The diuretic hormone receptor from membranous preparation of the malpighian tubules of *M. sexta* has been characterized [54], and two c-DNA clones have been isolated from the malpighian tubule membranes of *M. sexta* [51,52] and of the house cricket, *Acheta domesticus* [53], and expressed in a baculovirus system. The binding site of another neuropeptide hormone with diuretic activity, achetakinin, has been studied in the malpighian tubules of *A. domesticus* [13] and two other receptors for schistoFLRFamide have also been identified and characterized on the locust oviduct [60,61]. Recently, the bombyxin receptor has been studied in the ovaries of several moth species [20]. A tachykinin-receptor was recently cloned from the stable fly *Stomoxys calcitrans* [58] and immunolocalization of a tachykinin-receptor-like protein in the central nervous system of *Locusta migratoria* and *Neobellieria bullata* has been performed [59]; a leucokinin-binding protein has been characterized in *Aedes aegypti* malpighian tubules [43] as have two allatostatin receptors: one from *Drosophila melanogaster* [31] and the other from the cockroach *Diploptera punctata* midgut [10]. In an earlier study, another allatostatin receptor was identified in brain and corpora allata of the same cockroach [62]. To date, the pyrokinin/PBAN receptor from the pheromone gland has not been characterized. The presence of PBAN binding proteins (with approximate MWs of 100,000 and 115,000) in various tissues (brain-subesophageal complex, ventral nerve cord and thoracic muscle) of *Helicoverpa zea* has been reported using a photoaffinity labeling technique [19], however this study did not demonstrate its presence in the pheromone gland.

In a previous study [6] we reported on the development of a pyrokinin/PBAN radio-receptor assay (RRA) using a PBAN-derived radio-labeled ligand (^{3}H-tyrosyl-PBAN28–33NH_2). In that study we described the synthesis of the radio-ligand and determined preliminary conditions for its binding to sex pheromone gland membranes of *Heliothis peltigera* females. In the present study we have extended our examination of the ligand-receptor binding conditions, and used the assay for further characterization of the putative pyrokinin/PBAN receptor in the pheromone gland of *H. peltigera.*

2. Materials and methods

2.1. Insects

H. peltigera moths were reared on an artificial diet as described previously [15]. 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. Moth populations were refreshed every year with males caught from the wild by means of pheromone traps, as previously described [15]. New colonies were separated from existing ones and designated with consecutive numbers (GII, GIII, GIV, GV)

2.2. Peptide synthesis

Hez-PBAN (PBAN1–33NH_2) [49,50] and peptides derived from its sequence: PBAN9–33NH_2, PBAN13–33NH_2, PBAN19–33NH_2, PBAN26–33NH_2, PBAN28–33NH_2, PBAN9–18COOH and [Arg^{27},D-Phe^{30}]PBAN28–33NH_2 were synthesized on an ABI 433A automatic peptide synthesizer on Rink amide 4-methylbenzhydrylamine (MBHA) resin by means of the FastMoc^{TM} chemistry as described [63]. Backbone cyclic (BBC) peptides were synthesized by the Simultaneous Multiple Peptide Synthesis (SMPS) methodology on Rink amide MBHA Resin by means of 9-flourenylmethoxycarbonyl (Fmoc) chemistry as previously described [7]. Purity of the peptides was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) [7,63] and was found to be in the range of 90–95%. Purified peptides were characterized by time-of-flight mass spectrometry (TOF-MS) and amino acid analysis of hydrolyzates. Analytical methods and data of the linear and BBC peptides have previously been described [7,63]

Diiodo-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2 (diiodo-tyrosyl-PBAN28–33NH_2), which served as a basis for the preparation of the radio-labeled ligand was synthesized by the Merrifield Solid-Phase Peptide Synthesis method [9], on an Applied Biosystems peptide synthesizer Model 430A as described previously [22]. Peptide purity was assessed by RP-HPLC as described previously [1] and was found to be over 95%.

2.3. Preparation of a radio-labeled ligand

The radio-ligand that was used throughout the study was ^{3}H-tyrosyl-PBAN28–33NH$_2$ (specific activity: 12.4 mCi/μmol). The ligand was prepared by Rotem Industries Ltd. (Beer Sheva, Israel) by means of the gaseous tritiation method [44]. Radiochemical purity was determined by TLC on silica gel with n-butanol:acetic acid:water (50:25:25) and was found to be >95% [6]

2.4. Membrane preparation from pheromone glands

Glands were excised from 3.5-day-old (unless otherwise indicated) *H. peltigera* pheromone glands at the 8th –10th hour of photophase, and kept at -80°C until use. On the day of the experiment, glands were transferred to a glass-glass homogenizer containing one of the following ice-cold buffers: (*i*) 25 mM N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), pH 6.5 (preparation buffer I); (*ii*) 25 mM HEPES, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetra acetic acid (EGTA), 0.1 mM *o*-phenanthrolin and 0.01 mM phosphoramidon, pH 6.6 (preparation buffer II); (*iii*) 25 mM HEPES and a commercial protease inhibitor cocktail (Complete Mini and Complete Mini EDTA free, Boehringer Mannheim, Germany, one tablet per 10 ml of buffer) pH 6.5 (preparation buffers III and IV, respectively). Specific buffer numbers for each of the experiments described below are indicated in the Figure and Table legends. Homogenization was performed at a ratio of 10 glands/ml of buffer. Homogenates were centrifuged at 15,000× g in a fixed angle rotor at 4°C for 60 min, the supernatants were discarded and the pellet was resuspended in double-distilled water (DDW). Centrifugation was repeated twice and the resulting pellet was resuspended (at a ratio of 10 glands/ml) in DDW and tested for binding activity

Protein content was determined according to Bradford against a BSA standard curve [11]. Protein concentration ranged from 0.13 to 0.3 mg/ml.

2.5. Binding of ligand to gland membranes

One hundred μl of gland membranes (one gland equivalent/tube) were added to 50 μl 4× reaction buffer and 40 μl ^{3}H- PBAN28–33 NH$_2$ (10 pmol, total of 60,000 cpm) in a total reaction volume of 200 μl. Four reaction buffers were used in the binding experiments: Reaction buffer I, containing 10 mM HEPES, 10 mM $NaHCO_3$ and 145 mM sucrose, pH 8.0; Reaction buffer II: same as reaction buffer I, plus a cocktail of the following protease inhibitors: 0.1 mM PMSF, 0.1 mM EGTA, 0.1 mM *o*-phenanthrolin and 0.01 mM phosphoramidon, adjusted to pH 8.0; Reaction buffers III and IV: same as buffer I plus a commercial protease inhibitor cocktail (Complete Mini and Complete Mini EDTA free, respectively) adjusted to pH 8.0. The specific buffer used in each experiment is listed in the Figure and Table legends. Membranes were incubated for 75 min at room temperature. At the end of the incubation period 2 × 5 ml of ice-cold washing buffer (10 mM HEPES, 10 mM $NaHCO_3$, 5 mM NaCl, 39 mM KCl and 145 mM sucrose), pH 6.5, were added to each tube and the samples were filtered through a 25 mm GF/C filter (Whatman, Tamar, Jerusalem, Israel), pre-soaked for 1 h in washing buffer containing 0.005% Tween-20. The filters were dried at room temperature and transferred to 5 ml plastic vials. Four ml of scintillation liquid (Instagel, Packard) were added to each tube and the radioactivity was monitored with a Beckman Model LS 1701 scintillation counter. Each data point was determined in duplicate. Non-specific binding was determined in the presence of 100 μM PBAN1–33NH$_2$ (added in a volume of 10 μl). The choice of PBAN1–33NH$_2$ was based on our previous study that found this peptide to be slightly (7%) more effective than unlabelled PBAN28–33NH$_2$ in saturating the non-specific sites and displacing the radio-ligand [6]. Specific binding is defined as the difference between binding in the absence and presence of unlabeled PBAN1–33NH$_2$

Displacement experiments were performed similarly. Unlabeled peptides: PBAN1–33NH$_2$, PBAN9–33NH$_2$; PBAN13–33NH$_2$; PBAN19–33NH$_2$; PBAN26–33NH$_2$; PBAN28–33NH$_2$, [Arg^{27},D-Phe^{30}]PBAN28–33NH$_2$, PBAN9–18COOH and BBC peptides 25 and 27 [7] were added (at concentrations ranging from 0.01 to 1000 μM in a volume of 10 μl) to the incubation reaction, together with the ligand, and the experiment was performed as described above.

2.6. Pheromonotropic bioassay

The assay was performed essentially as previously described [2]. Briefly, *H. peltigera* females at various days post-emergence (as indicated for each individual experiment) were injected between the 4th and 7th hour of photophase with 2 μl of the tested peptide dissolved in 0.1 M sodium phosphate buffer, pH 7.4. Glands were excised 2 h post-injection and analyzed for pheromone content by capillary gas chromatography (CGC) as previously described [2]. All experiments were performed with 8–10 females per treatment

2.7. Statistical analysis

The results were subjected to one-way ANOVA. The significance of differences among means was evaluated with the Tukey-Kramer HSD test (unless otherwise indicated) at $P < 0.05$

3. Results

In a previous study [6] we described the synthesis of a radio-ligand peptide ^{3}H-tyrosyl-PBAN28–33NH$_2$ and de-

Table 1
Effects of buffers and pH on binding

Buffer	pH									
	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
Na Citrate	9 ± 3*	86 ± 3	81 ± 6							
Mes			100 ± 17	79 ± 9						
Na Phosphate			109 ± 12	104 ± 7	110 ± 5	119 ± 10				
HEPES				100	70 ± 13	104 ± 5	89 ± 25			
Mops				75 ± 15	100 ± 9	117 ± 11	107 ± 9			
Tris						43 ± 14*	2 ± 1*	2 ± 3*	3 ± 2*	
Na carbonate									48 ± 15*	47 ± 28*

Binding was carried out with pheromone gland membranes obtained from 3.5-day-old females. Membranes were made with preparation buffers II (see Methods) and binding was carried out in each of the above reaction buffers at 10 mM. Values represent specific binding expressed as percentages of the binding in the reference buffer (10 mM HEPES containing 145 mM sucrose, pH 6.5, defined as 100%). Numbers represent the mean ± SE of 2–6 samples obtained in two independent experiments. Statistical analysis was performed by the Dunnett test. An asterisk (*) indicates a significant difference in binding at $P < 0.05$.

termined its binding conditions with respect to the amount of biological material and incubation time, and examined factors affecting specific and non-specific binding. In the present study we have extended the examination of ligand-receptor binding conditions with respect to membrane preparation and incubation conditions (e.g. buffers at different pH values, divalent ions, and protease inhibitors), and applied the assay to the partial characterization of the pyrokinin/PBAN binding protein (hence receptor) in the pheromone gland of *H. peltigera.*

The first set of experiments involved examination of the effects of several buffers at various pH values on binding. Seven different buffers were used: Citrate, 2-[N-morpholino]ethanesulfonic acid (MES), sodium phosphate, HEPES, 3-[N-morpholino]propanesulfonic acid (MOPS), tris[hydroxymethyl]amino-methane (Tris) and sodium carbonate. As indicated in Table 1, binding did occur at a wide range of pH values (5.5–8.0) and did not differ significantly from that obtained in the reference buffer (10 mM HEPES buffer containing 145 mM sucrose, pH 6.5). At pH 5.0 binding was significantly lower (i.e. 9% as compared with 100% in the reference buffer). Binding at pH 8.5–9.5 was also lower than that in the reference buffer but the extent of binding depended heavily on the nature of the buffer itself. In sodium carbonate buffer at pH 9.0 and 9.5, binding was 48 and 47%, respectively, of that in the reference buffer, whereas in Tris buffer, binding was very low not only at pH 8.5 and 9.0 (2 and 3%, respectively) but also at pH 7.5 and 8.0 (43 and 2%, respectively) where binding in other buffers (i.e. sodium phosphate, HEPES and MOPS) was high. In our previous study we showed that the presence of $NaHCO_3$ had a positive effect on binding [6]. In the present study we tested its effect at a range of pH values, and found that the addition of 10 mM $NaHCO_3$ to HEPES buffer had the most pronounced effect increasing binding by 1.5-fold (at pH 6.5) and by 1.2-fold (at pH 8.0) compared with HEPES buffer solutions that did not contain the ion (data not shown). The absolute binding was highest at pH 8.0. Based on these results we chose the combination of 10 mM HEPES, 10 mM $NaHCO_3$ and 145 mM sucrose at pH 8.0 as the preferred reaction buffer for further studies.

The effects of chloride salts of divalent ions (Mg^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+}) on binding was also examined. The presence of Mn^{2+} or Mg^{2+} (at concentrations ranging from 1 nM to 1 mM)) did not have a noticeable effect on binding; nor did Ca^{2+} (at 25 and 50 mM). Zn^{2+} had a stimulatory effect but this resulted mainly from an increase in non-specific binding of the ligand to the membranous preparation.

Further examination of binding conditions addressed the effects of protease inhibitors on membrane preparation. Preparation of membranes from live tissues usually involves grinding, which releases proteases that may degrade membrane-associated proteins. In order to test possible involvement of proteases in the digestion of the PBAN binding protein we tested the effects of three protease inhibitor cocktails (at the membrane preparation stage). One mixture contained PMSF, EGTA, *o*-phenanthrolin and phosphoramidon (which inhibit serin and cystein proteases, thermolysin, carboxypeptidase Y and metalloproteases). The other two protease inhibitor cocktails were commercial mixtures (Complete Mini with and without EDTA), designed to inhibit a variety of enzymes such as trypsin, chymotrypsin, thermolysin, papain and other serine and cystein proteases including metalloproteases (in the case of the EDTA-containing mixture). All cocktails were added to 25 mM HEPES buffer, pH 6.5, which also served as a reference buffer, and binding was performed in all cases without protease inhibitors (in reaction buffer I which contained 10 mM HEPES, 10 mM $NaHCO_3$ and 145 mM sucrose, pH 8.0). As can be seen in Fig. 1, the various cocktails had differing effects on binding. The highest binding was obtained in the absence of inhibitors (preparation buffer I). Similar binding was obtained in the presence of the commercial inhibitor cocktail without EDTA (Complete Mini ETDA free, preparation buffer IV). Interestingly, the presence of EDTA in the same mixture of inhibitors (Complete Mini, preparation buffer III) resulted in a marked decrease

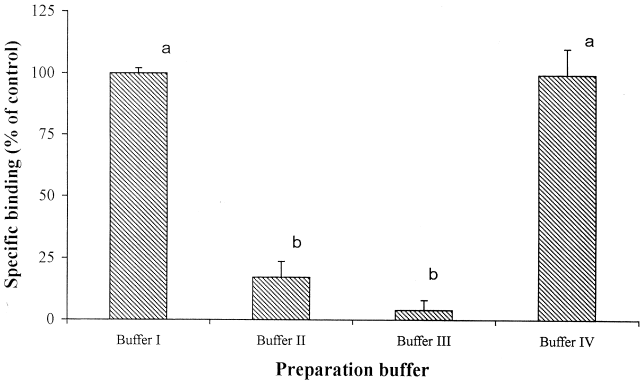

Fig. 1. Effects of protease inhibitors at the stage of membrane preparation on binding. Binding was carried out with pheromone gland membranes obtained from 3.5-day-old females. Membranes were made up with preparation buffers I-IV (see Methods) and binding was carried out with reaction buffer I. Specific binding is expressed as the ratio (as a percentage) between the amount of bound ligand in each of the preparation buffers and that in buffer I (defined as 100%). Values represent the mean ± SE of 3 samples and represent data obtained in two independent experiments. Bars with the same letter do not differ significantly at $P < 0.05$.

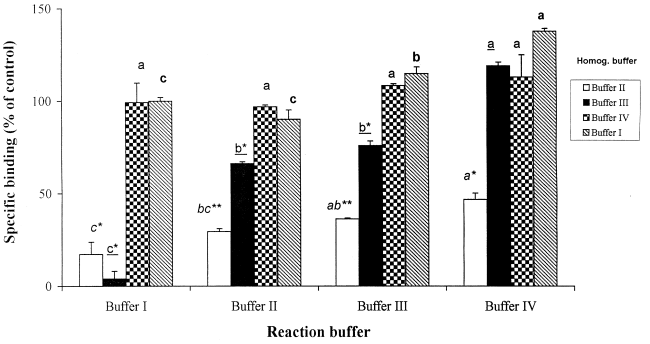

Fig. 2. Effect of protease inhibitors on binding. Binding was carried out with pheromone gland membranes of 3.5-day-old females. Membranes were made up with preparation buffers I-IV and binding was carried out with reaction buffers I-IV. Specific binding (for each membrane preparation) is expressed as the ratio (as a percentage) between the amount of bound ligand in each of the reaction buffers and that in reaction buffer I. Binding with membranes made up in preparation buffer I and incubated with the ligand in reaction buffer I was defined as 100%. Values represent the mean ± SE of 3 samples and represent data obtained in two independent experiments. Statistical analysis was performed for each membrane preparation in different reaction buffers (indicated by letters) and among the different membrane preparations in the same reaction buffer (indicated by asterisks). Bars with the same letter do not differ significantly at $P < 0.05$. An asterisk (*) indicates a significant difference in binding at $P < 0.05$.

in binding, and a similar result was obtained with preparation buffer II (which contained EGTA in addition to other protease inhibitors).

In the light of the unexpected effects of protease inhibitor cocktails that contained EDTA or EGTA we decided to test the effects of their presence in the reaction buffer. Membranes were prepared in the same four preparation buffers (I-IV) and each membrane preparation was tested for ligand binding in four different reaction buffers: reaction buffer I without inhibitors; reaction buffer II that contained PMSF, EGTA, *o*-phenanthrolin and phosphoramidon; and reaction buffers III and IV that contained the commercial mixture with and without EDTA, respectively. All reaction buffers were based on 10 mM HEPES, 10 mM $NaHCO_3$ and 145 mM sucrose, pH 8.0. Preparation buffer I and reaction buffer I were chosen as reference buffers and the extent of binding with these buffers was defined as 100%. As can be seen in Fig. 2 the effect of the protease inhibitors in the reaction buffer depended heavily on the preparation procedure. Membranes that were prepared in the absence of inhibitors (preparation buffer I) were only slightly affected by their presence in the reaction mixture. Membranes that were prepared in a buffer containing protease inhibitors without EDTA (preparation buffer IV) were not affected at all. The inhibitors, however, had a marked effect when added to the reaction mixture containing membranes that had been prepared in the presence of inhibitors (preparation buffers II and III and reaction buffers II, III and IV). The presence of a cocktail of inhibitors with no EDTA in the reaction mixture (reaction buffer IV) had the most profound effect on both membrane preparations, elevating binding (in the case of preparation buffer II in reaction buffer IV) to a level which did not differ significantly from that obtained with membranes prepared without inhibitors or in the presence of inhibitors without EDTA. Based on the above results we chose preparation buffer IV and reaction buffer IV as the preferred combination for all further experiments.

The development of the RRA allowed partial characterization of the biological and pharmacological properties of the pyrokinin/PBAN receptor in the sex pheromone gland of the female *H. peltigera*. The first series of experiments examined the dependence of ligand binding to *H. peltigera* pheromone gland membranes on age (i.e. days post-emergence). As can be seen in Fig. 3, binding did vary as a function of age, being very low at 0.5–3.5 days post-emergence and increasing gradually up to a maximum at 5.5 and 6.5 days. A significant decline was noticed at the age of 7.5 days.

In order to determine whether there was a correlation between the expression level of pyrokinin/PBAN receptor as determined by the RRA and the ability of the moths to synthesize sex pheromone in response to exogenously administered PBAN, *H. peltigera* females were injected with two doses of synthetic PBAN1–$33NH_2$ at various ages. The data revealed age-dependent differences (Fig. 4) where the amount of sex pheromone synthesized by 5.5-day-old females (in response to injection of 1 pmol PBAN1–$33NH_2$) was the highest, and dose dependency was exhibited only in 5.5- and 7.5-day-old females. In 1.5-and 3.5-day-old females, sex pheromone production was not dose dependent.

The RRA also enabled us to determine the receptor expression level in *H. peltigera* colonies that had been reared in our laboratory for 20, 12 and 8 life cycles (120, 72 and 48 weeks, respectively). As shown in Fig. 5, ligand binding decreased significantly with increasing age of the

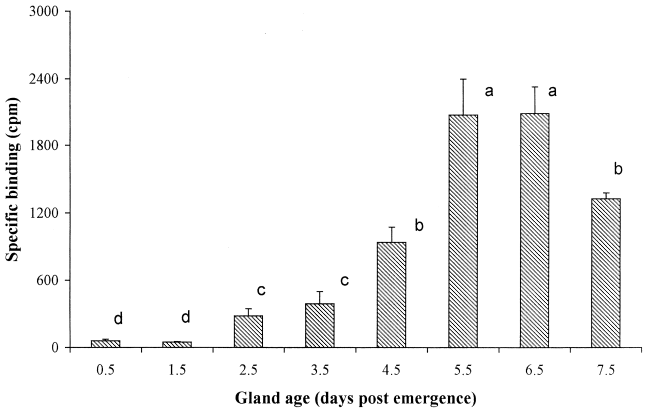

Fig. 3. ^{3}H-tyrosyl-PBAN28–33NH$_2$ binding as a function of age. Binding was carried out with pheromone gland membranes excised from females at different ages as indicated. Membranes were made up with preparation buffers IV and binding was carried out with reaction buffer IV. Specific binding is expressed as the difference between ligand binding in the absence and presence of 100 μM PBAN1–33NH$_2$. Values represent the mean ± SE of 4–8 samples. Bars with the same letter do not differ significantly at $P < 0.05$.

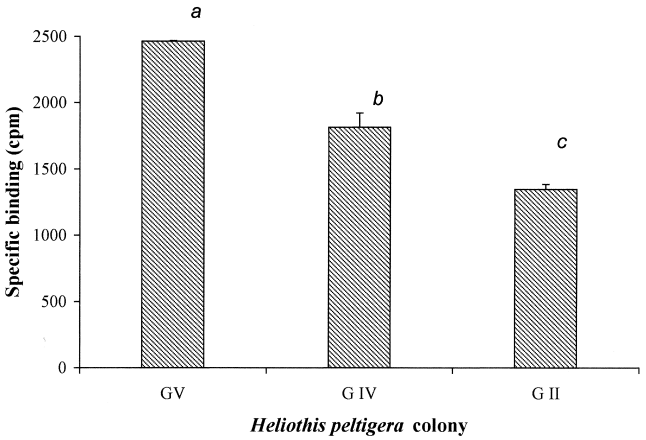

Fig. 5. ^{3}H-tyrosyl-PBAN28–33NH$_2$ binding as a function of the colony age. Binding was carried out with pheromone gland membranes excised from females at 5.5-days-post emergence. Membranes were made up with preparation buffers IV and binding was carried out with reaction buffer IV. GV, GIV and GII represent *Heliothis peltigera* colonies that were reared in the lab for 8, 12 and 20 life cycles, respectively. Specific binding is expressed as the difference between ligand binding in the absence and presence of 100 μM PBAN1–33NH$_2$. Values represent the mean ± SE of 3 samples and represent data that were obtained in two independent experiments. Bars with the same letter do not differ significantly at $P <$ 0.05.

colony, being highest in the youngest colony (reared for less than 1 year under laboratory conditions) and lowest in the older colonies. The response of the various colonies to injection of synthetic PBAN1–33NH$_2$ (at 1 pmol) was consistent with this trend, being lowest at GII (28 ng pheromone/gland) and highest at GV (221 ng pheromone/gland).

In our previous study [6] we demonstrated that the radioligand PBAN28–33NH$_2$ was displaced with unlabelled PBAN28–33NH$_2$ and PBAN1–33NH$_2$ with similar affinities (K_i values of 4.5×10^{-6} M and 3.9×10^{-6} M, respectively). In the present study we have extended the characterization of the pyrokinin/PBAN receptor by testing its ability to bind a variety of peptides derived from the PBAN sequence, and also their analogs. The peptides that were tested represented linear analogs of PBAN1–33NH$_2$ (PBAN9–33NH$_2$, PBAN13–33NH$_2$, PBAN19–33NH$_2$, PBAN26–33NH$_2$ and PBAN9–18COOH) all of which contained the C-terminal homologous sequence characteristic of the pyrokinin/PBAN family and exhibit variable degrees of pheromonotropic agonistic activity [2,3,5]; linear peptides with antagonistic activity ([Arg^{27},D-Phe^{30}]-PBAN 28–33NH$_2$) [8,63] and BBC peptides (BBC25 and BBC27) [7,8,23]. PBAN28–33NH$_2$ and PBAN1–33NH$_2$ served as reference peptide and Arg^{8}-Vasotocin served as a control unrelated peptide to demonstrate binding specificity of the

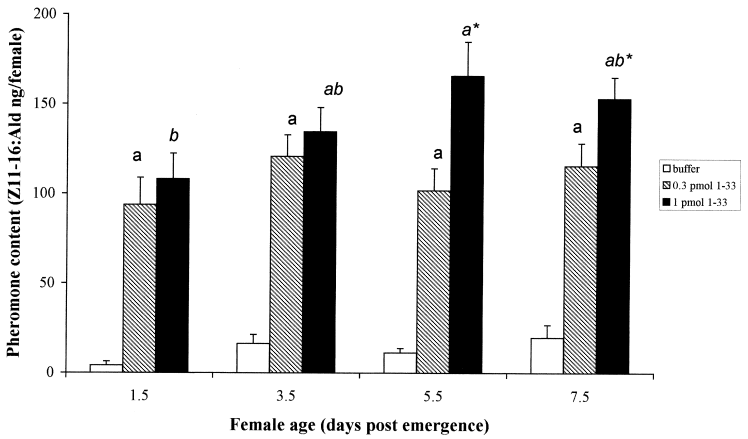

Fig. 4. Effect of synthetic PBAN1–33NH$_2$, injected at different ages, on pheromone production in female moths. PBAN1–33NH$_2$ was injected at a concentration of 0.3 or 1 pmol/2 μl, for 2 h. Injection of buffer was carried out similarly and served as a control. Glands were excised as described in Methods and the pheromone content was determined by GC analysis. Values represent the means ± SE of 8–10 samples. Statistical analysis was performed for each PBAN1–33NH$_2$ concentration among the various ages (indicated by letters) and between the two concentrations at each age (indicated by asterisks). Bars with the same letter do not differ significantly at $P < 0.05$. An asterisk (*) indicates a significant difference in binding at $P < 0.05$.

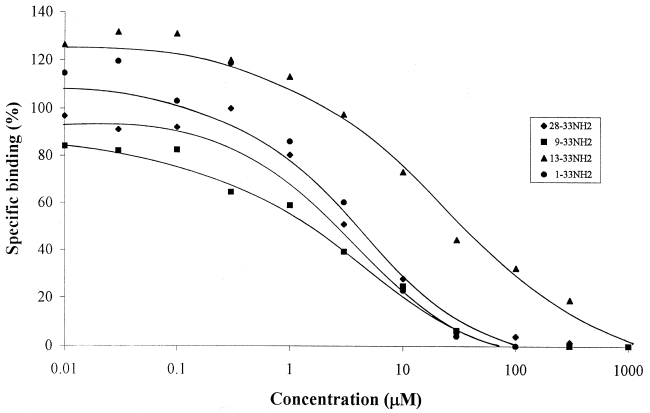

Fig. 6. Displacement of ^{3}H-labeled PBAN28–33NH$_2$ by unlabeled PBAN28–33NH$_2$, PBAN1–33NH$_2$, PBAN9–33NH$_2$ and PBAN13–33NH$_2$. Binding was carried out with pheromone gland membranes excised from females at 5.5-day-post emergence. Membranes were made up with preparation buffer IV and binding was carried out with reaction buffer IV. Non-specific binding was determined in the presence of 1 mM unlabeled PBAN1–33NH$_2$. Data represent the ratio (as percentages) of ligand binding in the presence of any tested concentration of the unlabeled peptides and in their absence. Binding in the absence of unlabeled peptides was defined as 100%. This figure represents the results of one out of three experiments, each of which resulted in similar displacement patterns.

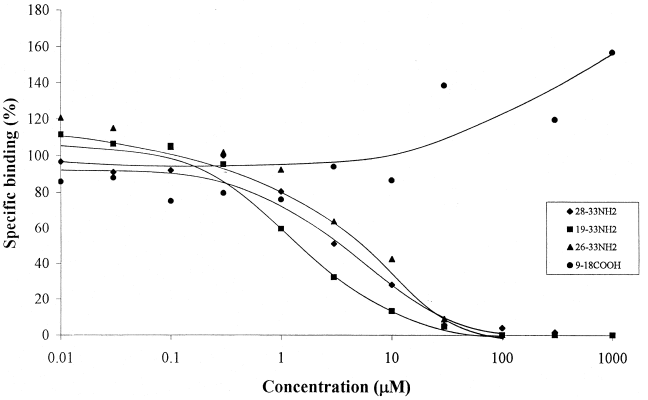

Fig. 7. Displacement of ^{3}H-labeled PBAN28–33NH$_2$ by unlabeled PBAN19–33NH$_2$, PBAN26–33NH$_2$ and PBAN9–18COOH. Displacement by unlabeled PBAN28–33NH$_2$ is presented as a reference. All other details are as described in the legend to Fig. 6.

pyrokinin/PBAN family. The results of the competition studies are shown in Figs. 6-8 and a summary of the inhibition constants (IC_{50}, representing the peptide concentration that displaced 50% of the bound ligand) are presented in Table 2. Since the concentration of the ligand employed in the present study was almost two orders of magnitude lower than the IC_{50}, it is valid to assume that this value represents the affinity of the peptide toward the receptor [32]. The data indicate that PBAN19–33NH$_2$ and PBAN9–33NH$_2$ were the most potent competitors for ^{3}H-tyrosyl-PBAN28–33NH$_2$ binding sites, followed by PBAN28–33NH$_2$ and PBAN1–33NH$_2$ that exhibited identical displacing potencies, and PBAN26–33NH$_2$ that was 2.3 times less potent than PBAN28–33NH$_2$.

Table 2
Inhibition constants (IC_{50}) of PBAN1–33NH$_2$ and PBAN–derived linear and BBC peptides

Peptide	IC_{50} (μM)
PBAN19–33NH$_2$	1.3
PBAN9–33NH$_2$	1.5
PBAN28–33NH$_2$	3
PBAN1–33NH$_2$	3
PBAN26–33NH$_2$	7
[Arg^{27},D-Phe^{30}]PBAN28–33NH$_2$	13
BBC25	24
BBC27	24
PBAN13–33NH$_2$	30
PBAN9–18COOH	>1000
Arg^{8}-Vasotocin	>300

IC_{50} values were determined from dose response curves (ranging from 0.01–1000 μM) and represent the peptide concentration that displaced 50% of the bound ligand. Binding was carried out with pheromone gland membranes obtained from 5.5-day-old females. Membranes were prepared with preparation buffer IV and binding was carried out with reaction buffer IV. Non-specific binding was determined in the presence of 1 mM unlabeled PBAN1–33NH$_2$. Binding in the absence of unlabeled peptides was defined as 100%.

The linear antagonist [Arg^{27},D-Phe^{30}]PBAN28–33NH$_2$ exhibited a low competing potency at the concentration at which agonistic peptides (i.e. PBAN1–33NH$_2$, PBAN9–33NH$_2$, PBAN19–33NH$_2$ and PBAN28–33NH$_2$) inhibited 50% of the radio-ligand binding (1.3–3 μM) and a 10-fold higher concentration was needed to replace 50% of the ligand. Similar results were obtained with the BBC antagonist 25 and the BBC compound 27. PBAN13–33NH$_2$ had the lowest competing activity and Arg^{8}-Vasotocin did not exert any competing activity up to a concentration of 300 μM. Interestingly, PBAN9–18COOH was also unable to displace the radio-ligand, but unlike the Vasotocin it stimulated binding at high concentrations (Fig. 7).

4. Discussion

PBAN is an insect neuropeptide of the pyrokinin/PBAN family, a major insect neuropeptide family known to regulate a variety of physiological and behavioral functions such as: sex pheromone biosynthesis in female moths, melanization and reddish coloration in moth larvae, egg diapause in the silkworm, contraction of the locust oviduct, myotropic activity of the hindgut in the cockroach and locust, and acceleration of pupariation in the fleshfly larvae. Despite the major importance of this family in the physiology of insects, very little information is available on the receptors that mediate these functions. Our present study involved the development of an RRA for the pyrokinin/PBAN family and its application to the partial characterization of this binding protein/receptor.

In a previous study [6] we concentrated on the first part of the RRA development process i.e. selection and labeling

of a ligand, and determination of preliminary reaction parameters (e.g. time, amount of biological material, ligand concentration, etc.) In the present study, we have extended the development of the RRA by establishing improved binding conditions that enabled partial characterization of the putative pyrokinin/PBAN receptor in the pheromone gland of *H. peltigera*. Development of the RRA in both studies was based on a radio-labeled hexapeptide ligand (^{3}H-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2), which constitutes the C-terminal homologous sequence shared by all neuropeptides of the pyrokinin/PBAN family.

Three major factors were tested for their effects on binding: buffers at different pH values, divalent ions and presence of protease inhibitors at the stage of membrane preparation and during ligand-receptor binding. These experiments revealed that ligand-receptor interaction occurs over a wide pH range (5.5–8.0) whereas lower (e.g. 5.0) and higher (8.5–9.5) pH values had negative effects on binding (Table 1). Binding of the ligand to the PBAN receptor was found to depend not only on pH but also on the nature of the buffer. Tris-based buffers caused a significant decrease in binding, even at pH values for which binding was high, whereas $NaHCO_3$ promoted binding even at high pH values. Addition of various divalent ions to the incubation mixture (Mn^{2+}, Mg^{2+}, Zn^{2+} and Ca^{2+}) did not have any effect on the ligand-receptor interaction and similar results were obtained previously with the monovalent ions Na, K and Cl [6]. The effect of divalent ions was studied in other insect neuropeptide receptor systems and revealed inverse results. In the study of the AKH receptor of the fat body binding was found to be heavily dependent on the presence of Ca and Mg [64], whereas in the study of the diuretic hormone receptor of malpighian tubules Ca and Mg were found to inhibit the binding of the ligand [54].

Despite their lack of a direct effect on binding, ions do have some indirect effects. This assumption is based on the fact that the presence of ion chelators EDTA, EGTA or *o*-phenanthrolin at the stage of membrane preparation resulted in a marked decrease in binding (Fig. 1). Interestingly, this effect could be reversed by the presence of protease inhibitors (whether or not they contained these chelators) in the binding reaction (Fig. 2). At present, we have no comprehensive explanation for the strong inhibitory effect of these chelators. One possible explanation is that the chelators remove ions that are essential for stabilization of the membranes causing the membranes to be more susceptible to proteases. Protease inhibitor cocktails have been used in other studies that employed epithelial cells as a source of membrane preparation. Mazzocco-Manneval et al., [37] used a mixture of PMSF, EGTA and *o*-phenanthrolin in the course of membrane preparation and storage of cockroach midgut and hindgut proctolin receptor; a similar composition (EGTA and *o*-phenanthrol) was used by Bowser and Tobe [10] for the preparation of the allatostatin receptor of midgut plasma membranes; and Pietrantonio et al. [43] used Complete^{TM} protease inhibitor tables for preparation of the leucokinin receptor of malpighian tubules. In all of the above studies protease inhibitors were also present during the binding assay. Although protease inhibitors were found to play a major role in some of the studies (e.g. the allatotropin receptor of the cockroach midgut; [10]), these studies did not discriminate between the protective effect of inhibitors in the course of the membrane preparation process and that during the binding assay itself.

The development of the above RRA proved the existence of a putative pyrokinin/PBAN receptor in the sex pheromone gland of the female *H. peltigera* moth, and allowed partial characterization of its biological and pharmacological properties. The biological characterization involved examination of the expression level of the receptor in females of various ages post-emergence and in *H. peltigera* moth colonies that had been reared in the laboratory for differing lengths of time. The assay was also used to determine the presence of pyrokinin/PBAN binding in pheromone glands of two other moth species

Indeed, application of the RRA revealed the presence of pyrokinin/PBAN receptor in other moths (*Helicoverpa armigera* and *Spodoptera littoralis*) (data not shown). It also showed that moths reared for a long periods in the laboratory (over 1 year) exhibited a significant decrease in the receptor expression or ability to bind ligand (Fig. 5); and that receptor expression varied as a function of age, reaching a maximum at 5.5–6.5 days post-emergence (indicated by a high degree of ligand binding at this age) (Fig. 3).

The expression levels of the receptor (as indicated by the binding studies) in the various moth colonies and at different ages post-emergence correlated well with the responses of the colonies to the injection of synthetic PBAN1–$33NH_2$, thus, hinting at the possibility that the RRA indeed determines the receptor that is activated in vivo by PBAN1–$33NH_2$. This notion is further strengthened by the pharmacological characterization of the receptor (see below). Examination of the age-dependence of the pheromone biosynthesis elicited by exogenously administered PBAN and of the pheromone production that results from the endogenous "natural" mechanism in the female moth at the same ages revealed a good correlation. Studies performed by Dunkelblum and Kehat [16] on the age dependence of the calling behavior and pheromone content in *H. peltigera* females revealed low content up to 3.5 days and a gradual increase up to a maximal activity at 5.5–6.5 days.

The development of the RRA also allowed partial pharmacological characterization of the pyrokinin/PBAN receptor, and included determination of the binding affinity of various peptides derived from the PBAN sequence, and of their analogs. Eleven different peptides were tested for their ability to compete with the radio-labeled ligand (Figs. 6–8 and Table 2): six linear C-terminally amidated peptides derived from the sequence of PBAN1–$33NH_2$, including unlabeled PBAN28–$33NH_2$, which is homologous to the ligand; one linear acid-free peptide; two antagonists one of them linear and the other BBC and a "non-relevant" control

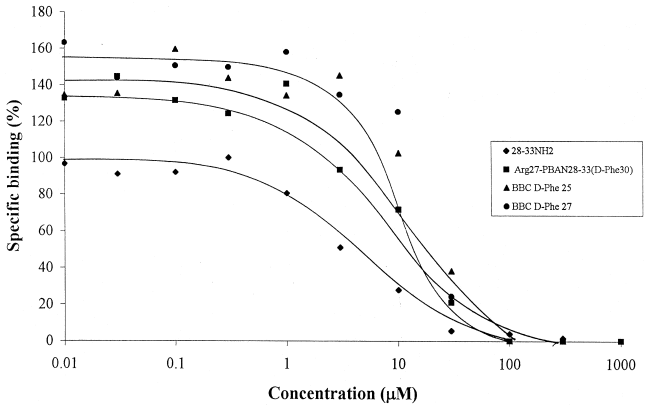

Fig. 8. Displacement of ^{3}H-labeled PBAN28–33NH$_2$ by unlabeled [Arg^{27},D-Phe^{30}]PBAN28–33NH$_2$ and the BBC peptides 25 and 27. Displacement by unlabeled PBAN28–33NH$_2$ is presented as a reference. All other details are as described in the legend to Fig. 6.

peptide (Arg^{8}-Vasotocin) which does not belong to the pyrokinin/PBAN family. The peptides PBAN1–33NH$_2$, PBAN9–33NH$_2$, PBAN19–33NH$_2$ and PBAN28–33NH$_2$ displaced the radio-ligand at comparable potencies; PBAN26–33NH$_2$ was slightly less potent, and PBAN13–33NH$_2$ was the weakest among the agonistic linear amidated peptides. The identical displacement curves obtained with PBAN1–33NH$_2$ and PBAN28–33NH$_2$ (K_i value of 3×10^{-6} M), and the correlation between the binding affinities of the tested peptides (i.e. PBAN9–33NH$_2$, PBAN19–33NH$_2$ and PBAN28–33NH$_2$, Table 2), and their bioactivities as indicated by the in vivo pheromonotropic bioassay [2,3,5] support the notion that the receptor that was characterized in the present study is the putative pyrokinin/PBAN receptor. Another peptide that exhibited correlation was PBAN9–18COOH. The peptide did not displace the ligand up to 10 μM but stimulated binding at higher concentrations (Fig. 7). A previous examination of the in vivo pheromonotropic activity of this peptide revealed synergism with PBAN1–33NH$_2$ (data not shown). The only peptide that did not exhibit a good correlation in both assays was PBAN13–33NH$_2$. This peptide exhibited a much lower affinity toward the receptor in the RRA (as compared with the other linear agonistic peptides) and a high pheromonotropic in vivo activity (that was even slightly higher than that of PBAN1–33NH$_2$ and PBAN28–33NH$_2$) [7]. The reason for this mismatch is not clear at present. The specificity of the assay to the pyrokinin/PBAN family of neuropeptides was demonstrated by the inability of Arg^{8}-Vasotocin to displace the radio-labeled ligand.

Interestingly, the two antagonistic peptides [Arg^{27},D-Phe^{30}]PBAN28–33NH$_2$ and BBC peptide 25 had relatively low displacing activities, despite their high antagonistic potency [7,8,23,63]; furthermore their affinity did not differ from that of the BBC peptide 27 which was devoid of any agonistic or antagonistic activity [7,8]. These data hint at the possibility that these antagonists are not competitive, i.e. that they do not bind to the same site as the agonist but exhibit their inhibitory activity by means of allosteric effects thus, in the case of the BBC peptides, both compounds (BBC 25 and 27) probably bind to the "receptor vicinity," but only one (BBC 25) has an allosteric effect that may inhibit ligand binding.

Despite the good overall correlation between the pharmacological properties and the bioactivity of most of the tested peptides and the fact that the binding site that has been characterized meets the physiological criteria for the definition of a receptor, based on its saturability, reversibility and specificity, it should be kept in mind that it exhibited a low affinity, having a K_d value of $5.73 \pm 1.05 \times 10^{-6}$ M and a Bmax of 1.85 ± 0.22 nmol/mg protein [6]. As previously suggested the putative pyrokinin/PBAN receptor may exhibit a "spare receptor" behavior, with a full response being obtained at a low ligand capacity [32]. It is, however, possible that the binding site that has been characterized is not the only pyrokinin/PBAN receptor and that there are other receptors on the pheromone gland epithelial cells that bind PBAN1–33NH$_2$ and other PBAN-derived peptides with higher affinities. In this context it is important to note that the pheromonotropic in vivo potency of synthetic PBAN28–33NH$_2$, i.e. the concentrations that were needed to induce sex pheromone activity ranged from 10 pmol/female (5×10^{-6} M) [2] to 100-1000 pmol of peptide/female (10^{-5} to 10^{-4} M) [48]. Although these concentrations comply with the high K_d value that was obtained in our study, such a comparison may be imprecise as it does not reflect the effective concentration of the injected peptide at the target site due to effects of diffusion, enzymatic degradation, clearance, etc.

In summary, the RRA that was developed in the present study proved the existence of a pyrokinin/PBAN receptor in the sex pheromone gland of the moth *H. peltigera* and facilitated the partial characterization of the expression and of some biological and pharmacological properties of this receptor. It is envisaged that the information that was obtained in the course of the present study, as well as the availability of the RRA, will lead to a better insight into the physiology of the pyrokinin/PBAN family, and to a better understanding of the correlation between the biological activities of the various peptides and their receptors. This RRA can also serve as a basis for the development of an HTSA for screening chemical and biological combinatorial libraries. This aspect is of importance for the discovery of pyrokinin/PBAN agonists and antagonists, which can be further developed to provide highly selective and environmentally friendly insecticides and insect control agents.

Acknowledgments

We are grateful to Dr. Hillary Voet, Faculty of Agricultural, Food and Environmental Studies, Hebrew University of Jerusalem for the helpful discussions and assistance in the statistical analysis of the data. We would like to thank

Mrs. Irit Schefler, from the Dept. of Entomology, The Volcani Center, ARO, Bet Dagan, for performing the gas chromatography analysis. This research was supported by the Israel Science Foundation, administered by the Israel Academy of Sciences and Humanities (to M.A.) and by the Israel Ministry of Science and Technology (to M.A. and C.G.).

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