Pheromonotropic and melanotropic PK/PBAN receptors: Differential ligand–receptor interactions

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The aim of the present study was to further characterize the PK/PBAN receptors and their interaction with various PK/PBAN peptides in order to get a better understanding of their ubiquitous and multifunctional nature. Two cloned receptors stably expressed in Spodoptera frugiperda (Sf9) cells were used in this study: a Heliothis peltigera pheromone gland receptor (Hep-PK/PBAN-R) (which stimulates sex pheromone biosynthesis) and Spodoptera littoralis larval receptor (Spl-PK/PBAN-R) (which mediates cuticular melanization in moth larvae) and their ability to respond to several native PK/PBAN peptides: β-subesophageal neuropeptide (β-SGNP), myotropin (MT) and Leucophaea maderae pyrokinin (LPK), as well as linear and cyclic analogs was tested by monitoring their ability to stimulate Ca²⁺ release. The receptors exhibited a differential response to β-SGNP, which activated the Hep-PK/PBAN-R but not the Spl-PK/PBAN-R – a response opposite to that previously demonstrated with diapause hormone (DH). MT was somewhat more active on Spl-PK/PBAN-R than on Hep-PK/PBAN-R. LPK elicited similar positive responses in both receptors (like that with PBAN). A differential response toward both receptors was also noticed with the PBAN-derived backbone cyclic (BBC) conformationally constrained peptide BBC-5. The peptides BBC-7 and BBC-8 activated both receptors. The results correlate between two PK/PBAN mediated function (cuticular melanization and sex pheromone biosynthesis) and the peptides that activate them and thus advance our understanding of the mode of action of the PK/PBAN family, and might help in exploring novel high-affinity receptor-specific antagonists that could serve as a basis for development of new families of insect-control agents.

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Introduction

The pyrokinin/pheromone biosynthesis-activating neuropeptide (Np) family is one of the most studied neuropeptide (Np) families in insects, and one of the most important in regulating their physiology. The family is ubiquitous and multifunctional, and plays major roles in regulating a wide range of developmental processes: feeding, mating and behavior [4]. The family comprises the following C-terminally amidated peptides: PKs, PBANs, diapause hormone (DH), β- and γ-subesophageal Nps (SGNPs), and myotropins (MTs), all of which share a common C-terminal sequence (FXPRLa). PK/PBAN peptides are encoded by two genes: pban gene, which in moths encodes five peptides, four of which contain the C-terminal consensus sequence FXPRLa (PBAN, DH, β- and γ-SGNPs); and capa gene, which encodes three peptides, one of which – a DH-like peptide (termed herein CAPA-DH) – is a member of the family. To avoid terminological confusion, in the present paper we refer to the individual peptides as pban-gene-encoded peptides or capa-gene-encoded peptides and to all the FXPRLa-containing peptides as the PK/PBAN family. For a review on the PK/PBAN peptides see [4,15].

The ubiquitous and multifunctional nature of the PK/PBAN peptide family raises many questions regarding the mechanisms by which these Nps elicit their effects in different insects, the nature of the receptors that mediate these functions, and also the nature of the receptor/ligand interactions and their translation to downstream cellular signaling. Similarly to other Nps, the PK/PBAN Nps activate cellular processes via G-protein-coupled receptors (GPCRs) [15] located on the surface of the Nps’ target cells [1]. In the past decade many PK/PBAN-Receptors (PK/PBAN-Rs) have been cloned from pheromone glands of adult female moths of various species (e.g., Helicoverpa zea, Bombyx mori, Heliothis virescens, Heliothis peltigera, Helicoverpa armigera, Plutella xylostella, Spodoptera exigua, and Pseudoleitia separata), moth larvae (of, e.g., Spodoptera littoralis and H. virescens), and pupae of B. mori and Ostrinia
nubilalis [7,8,12,14–16,18,20,24,30]. Occurrence of PK/PBAN-R in adult male moths has also been reported [5]. PK/PBAN receptors have also been cloned from several non-Lepidopteran insects such as Drosophila melanogaster, Anopheles gambiae, Aedes aegypti and Rhodnius prolixus [6,10,21,22,27].

More detailed studies have recently revealed the presence of multiple isoforms or variants of PK/PBAN-Rs of the moth pheromone gland in H. virescens, B. mori, H. armigera, H. zea, P. separata, Manduca sexta, and O. nubilalis; these differ in having a “long” or “short” C-terminal tail, and arise from alternative splicing [4,16,18,20]. To date, about 40 PK/PBAN-Rs, including different variants, have been cloned from various insects, and most of them were transiently or stably expressed in diverse expression systems, e.g., insect or mammalian cell lines or Xenopus oocytes. A few others have been annotated according to sequence homologies with previously cloned PK/PBAN-Rs or predicted sequences [4,10,18,20,22]. Many of the actually cloned receptors have been identified, mainly by their ability to elicit Ca flux in response to stimulation with various PK/PBAN ligands.

Although much progress has been made on characterization of the PK/PBAN-Rs, we still lack information on their interactions with the peptides of this family, and very little is known about the correlation between such interactions and their in vivo bioactivity. This issue is of major importance, especially in view of the multifunctional nature of this family of Nps. Currently, it is still not clear which receptor(s) mediate each of the in vivo functions and which peptide elicits it; we still do not know whether each function is mediated by a single or multiple receptor(s), or whether each function is elicited by one or more PK/PBAN peptides. All of the above issues require further investigation and need to be examined in the context of their in vivo functions. Analysis of the interaction of receptors that mediate known functions (i.e., sex pheromone biosynthesis and cuticular melanization) with different peptides of the PK/PBAN family as described below may shed light and provide answers for some of these issues. In the present paper the receptors are referred to by the general term: PK/PBAN-R and are designated according to terminology suggested by Raina and Gade for insect Nps [25] (e.g., Drm-R for D. melanogaster receptor).

In previous studies we used in vivo pheromone and melanotropic assays (with H. petelgira and S. littoralis, respectively) to investigate the structure–activity relationship of a large variety of PK/PBAN peptides and their analogs, and to determine whether the two functions were mediated by different PK/PBAN-Rs that responded to the different peptides. For this purpose, we used conformationally constrained backbone cyclic (BBC) PK/PBAN agonists and antagonists, which exhibit high receptor selectivity [2]. These studies yielded strong indications of the existence of two different PK/PBAN-Rs in H. petelgira and S. littoralis, one which mediates sex pheromone biosynthesis in the pheromone gland and another that mediates cuticular melanization in moth larvae [3]. To enable further study of this issue the pheromone gland receptor of H. petelgira (Hep-PK/PBAN-R) (which mediates sex pheromone biosynthesis in female moths) and the larval receptor of S. littoralis (Spl-PK/PBAN-R) (which, most likely, mediates cuticular melanization in larvae) were cloned and stably expressed in Spodoptera frugiperda (SF9) cells, and their structure, electrostatic potentials and cellular functional properties (i.e., induction of Ca^{2+} flux) in response to stimulation with PBAN and DH ligands were examined [12]. These studies indicated structural and electrostatic differences as well as differences in binding of the two ligand peptides, thus further strengthening the notion that different in vivo functions of PK/PBAN peptides may be mediated by different PK/PBAN-Rs and elicited by (a) different peptide(s). The aim of this study was to further prove this issue with additional native and synthetic conformationally constrained peptides. Toward this goal we expanded the analysis and tested the ability of the cloned Hep-PK/PBAN and

![Fig. 1. Structure of BBC peptides 5, 7 and 8.](image)

Spl-PK/PBAN receptors to respond to: additional native PK/PBAN peptides (β-SGNP, MT and LPK); peptides derived from the Hez-PBAN sequence (PBAN28–33 and C-terminally free acid PBAN); and their analogs (BBC-5, BBC-7, and BBC-8). The findings indicated, once again, the differences between the two receptors as reflected in their binding activities following activation by the above peptides.

**Materials and methods**

**Peptides**

Synthetic PBAN – based on the sequence of H. zea PBAN (Hez-PBAN), free acid Hez-PBAN (PBAN-COOH), the hexapeptide PBAN28–33-amide derived from the C-terminus of Hez-PBAN, S. littoralis DH (based on the sequence of S. littoralis DH), Pseudalea separata β-SGNP (previously termed pheromonotropin, PT), Leucopephae maderae pyrokinin (LPK) and Locusta migratoria myotropin (MT) – were synthesized with an ABI 433A automatic peptide synthesizer on Rink amide MBHA resin by means of the FastMoc chemistry as described previously [29] (see Table 1 for amino acid sequences). BBC peptides 5, 7, and 8 (see Fig. 1) were synthesized as previously described [2].

**Cell lines**

S. frugiperda pupal ovarian cell line (SF9) [28] and SF9 cells stably transfected with either S. littoralis PK/PBAN-R (Spl–PK/PBAN-R) [30] or with H. petelgira PK/PBAN-R (Hep-PK/PBAN-R) [12] were cultured at 27 °C in 4 mL of Grace’s Medium (Biological Industries, Beit-haEmek, Israel) supplemented with 10% heat-inactivated fetal bovine serum, 1% Lactalbumin, 1% Yeastolate, 0.5% Penicillin G, and 0.5% Streptomycin (all supplied by Biological Industries and referred to below as complete Grace’s insect medium) in 25-cm² flasks (Corning, NY, USA). Cell passage was performed every 3–4 days.

**Measurement of intracellular calcium level**

Cells were grown overnight in 25-cm² flasks, as described in Section “Cell lines”. On the day of the experiment 4 × 10⁶ cells of each of the following lines were harvested and transferred into 15-mL glass tubes: control untreated SF9 cells; cells transfected with an ‘empty’ pL2/V5-His vector; and SF9 cells stably expressing Hep-PK/PBAN-R or Spl-PK/PBAN-R. Growth medium was removed by centrifugation at 800 × g for 10 min, the pellet was re-suspended in 4 mL of cold PBS at pH 7.2, and centrifuged again under the same conditions. The pellet then was re-suspended in 4 mL of Fluo-4 AM (Molecular Probes, Carlsbad, CA, USA), at a final concentration of 2 μM, made up in modified Ringer’s buffer (12 mM NaCl, 21 mM KCl, 170 mM glucose, 3 mM CaCl₂, 18 mM MgCl₂, and 10 mM PIPES, at
pH 7.4) containing 1 mM Probenecid and 0.01% Pluronic F-127 (both purchased from Sigma and made up in modified Ringer’s buffer). Cells were incubated for 45 min on a rotating shaker at a low speed at room temperature in darkness. After incubation the cells were washed twice with 4 mL of Ringer’s modified buffer, followed each time by centrifugation at 800 x g for 10 min, re-suspended in 4 mL of Ringer’s modified buffer and kept in darkness for another 30 min on a rotating shaker, to allow hydrolysis of the Flu-4-AM ester bond. Cell suspension aliquots of 80 µL, containing approximately 10⁵ cells, were placed in each well of a black 96-well plate with a clear bottom (Greiner, Frickenhausen, Germany) and 20 µL of 5 x concentrated peptides made up in Ringer’s modified buffer were injected into each well at the final concentrations indicated in the figures. At the end of the experiment Ionomycin (Alomone Laboratories, Jerusalem, Israel), made up in 0.1 M glycine buffer at pH 10.0, was added to separate wells at a final concentration of 10 µM in order to determine the maximal positive Ca²⁺ influx response. Each peptide was tested in quadruplicate at each concentration. Fluorescence was measured with a Tristar LB 941 plate reader (Berthold Technologies, Bad Wildbad, Germany) with a 485-nm excitation filter and the signal was monitored via a 535-nm emission filter. Each well was monitored separately. Fluorescence was monitored every second for 5 s prior to addition of the tested peptide or ionomycin, to determine the baseline, and every second for 2 min following addition of the peptide or ionomycin. The results are presented as the ratio (expressed as a percentage) between the signal obtained at each peptide concentration and that of ionomycin after subtraction of the background (baseline) value.

**Statistical analysis**

All results were subjected to one-way ANOVA. The significant differences among means were evaluated by application of the Tukey–Kramer honestly significant difference (HSD) test at p < 0.05. The data are presented as means ± SEM.

**Results**

In order to evaluate the cellular functional differences between the Hep- and the Spl-PK/PBAN-Rs the cloned receptors were stably expressed in S99 cells and dose–response experiments were carried out in which we monitored free intracellular Ca²⁺ elicited by stimulation with a variety of PK/PBAN peptides (β-SGNP, previously termed pheromonotropin, LPK, MT) and their analogs (a free-acid PBAN analog, i.e., PBAN-COOH; a PBAN C-terminal-derived hexapeptide, i.e., PBAN28–33 (YFSPRL-amiide) and three BBC peptides (BBC-5, BBC-7, and BBC-8) [2].

Stimulation of both receptors with β-SGNP at concentrations ranging from 0.00001 M up to 1000 M revealed a differential response. The Hep-PK/PBAN-R exhibited a strong response to the peptide over a concentration range of 0.001–0.1 M exhibiting 50, 55, and 38% activity, respectively, compared with that obtained with 10 µM ionomycin (defined as 100%). No response was obtained with the Spl-PK/PBAN-R at β-SGNP doses of 0.001 M or higher (up to 1000 M) (Fig. 2A). β-SGNP evoked a very weak Ca²⁺ flux at the tested concentrations, with a maximal activity of 28% at 0.001 M when applied on the Spl-PK/PBAN-R (Fig. 2B). The EC₅₀ values obtained with this peptide with Hep–PK/PBAN-R and Spl-PK/PBAN-R were 0.001 M and >1000 M, respectively (Table 2). MT also differed in its activity on both receptors, however, in contrast to β-SGNP, MT activated both receptors but was more active on Spl-PK/PBAN-R than on Hep–PK/PBAN-R, and it stimulated Ca²⁺ flux at a much lower concentration than that required to

**Table 1**

| Amino acid sequence of PK/PBAN family peptides used in this study. |
|--------------------------|--------------------------|
| Code name | Insect species          | Amino acid sequence                  |
| PBAN      | Helicoverpa zeae        | LSDDMPATPADQEMYRQDPIDRSKYESPRL-amiide |
| PBAN-COOH | Helicoverpa zeae        | LSDDMPATPADQEMYQPIDPEESKYESPRL-amiide |
| PBAN 28–33| Helicoverpa zeae        | AMSGDRGSKYESPRL-amiide                |
| DH        | Spodoptera littoralis   | YFSPRL-amiide                        |
| β-SGNP    | Pseudaletia separata    | NERDGGSKYESPRL-amiide                |
| LPK       | Leucophaea maderae      | KSLDDKVFENVEPRL-amiide               |
| MT-II     | Locusta migratoria      | pETSFUPRL-amiide                     |

PBAN-COOH: free-acid PBAN; PBAN28–33: hexapeptide derived from the C-terminus of PBAN; DH: diuretic hormone; β-SGNP: β subesophageal ganglion neuropeptide (also known as pheromonotropin); LPK: pyrokinin; MT: myotropic hormone. Underlined amino-acids indicate the consensus sequence.
stimulate the Hep-PK/PBAN-R. The response of Spl-PK/PBAN-R to MT was maximal at 0.01 pM, at which its activity reached 64% whereas the response of the Hep-PK/PBAN-R to the peptide resulted in a maximal activity of only 40% at 0.1 pM (Fig. 3A and B). The EC50 values for both receptors were 0.0015 pM and >1000 pM, respectively (Table 2). Unlike β-SGNP and MT, LPK was equipotent toward the two receptors; it stimulated both receptors over the same concentration range (0.01–10 pM), to similar extents (42, 57, 59 and 56% and 36, 50, 61 and 38%, with Hep-PK/PBAN-R and Spl-PK/PBAN-R, at 0.01, 0.1, 1 and 10 pM, respectively) (Fig. 4A and B) and exhibited similar EC50 values (of 0.05 and 0.1 pM, respectively) (Table 2). LPK did not elicit a significant response from either receptor at doses of 100 pM or 1000 pM. The C-terminal-derived peptide PBAN28–33, which comprises the active site of the PK/PBAN, exhibited no stimulatory activity with the Hep-PK/PBAN-R over a concentration range of 1 pM–10 μM, and showed very low activity with Spl-PK/PBAN-R (Data not shown). The C-terminally free-acid PBAN molecule exhibited no activity over a concentration range of 1 nM–1000 μM (Data not shown). The EC50 values for both peptides were >10 μM and >1000 pM, respectively (Table 2).

Similar analyses were carried out with three BBC peptides. BBC-5 was devoid of any activity on the Hep-PK/PBAN-R at a concentration range of 0.001 pM to 1000 pM but stimulated the Spl-PK/PBAN-R to an extent of 39% (Fig. 5A and B). The EC50 values for both receptors were >1000 pM (Table 2). In contrast, BBC-7 activated both receptors, although its activation of Hep-PK/PBAN-R was higher (60% at 0.1 pM) than that of Spl-PK/PBAN-R, which reached 48% at 0.01 pM and extended over a much broader concentration range (Fig. 6A and B). The EC50 values for the receptors were 0.015 pM for Hep-PK/PBAN-R and >1000 pM for Spl-PK/PBAN-R (Table 2). No significant response was obtained from Hep-PK/PBAN-R or Spl-PK/PBAN-R at BBC-7 doses of 1 pM or higher (up to 1000 pM). Interestingly, BBC-8 was the most active peptide amongst all tested compounds; it activated both receptors at concentrations in the nM range, with activity reaching 69% of that of ionomycin, at 1 nM, on the Hep-PK/PBAN-R and was also active over a wide range of concentrations (1 nM–10 μM) with maximal activity at the lowest tested concentration of all peptides. BBC-8 was active on Spl-PK/PBAN-R, over the same concentration range (Fig. 7A and B); the EC50 values of BBC-8 with both receptors were <1 nM (Table 2). No response was obtained with Hep-PK/PBAN-R or Spl-PK/PBAN-R at BBC-8 doses of 0.1 pM or higher (up to 1000 pM).

**Table 2**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Hep-PK/PBAN-R</th>
<th>Spl-PK/PBAN-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAN</td>
<td>0.15 pM</td>
<td>0.12 pM</td>
</tr>
<tr>
<td>PBAN-COOH</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>β-SGNP</td>
<td>0.001 pM</td>
<td>No effect</td>
</tr>
<tr>
<td>PBAN28–33</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>DI</td>
<td>0.013 pM</td>
<td>No effect</td>
</tr>
<tr>
<td>LPK</td>
<td>0.05 pM</td>
<td>0.1 pM</td>
</tr>
<tr>
<td>MT</td>
<td>&gt;1000 pM</td>
<td>0.0015 pM</td>
</tr>
<tr>
<td>BBC-5</td>
<td>No effect</td>
<td>&gt;1000 pM</td>
</tr>
<tr>
<td>BBC-7</td>
<td>0.015 pM</td>
<td>&gt;1000 pM</td>
</tr>
<tr>
<td>BBC-8</td>
<td>&lt;1 nM</td>
<td>&lt;1 nM</td>
</tr>
</tbody>
</table>

Values are based on the data presented in Figs. 2–7. Values represent the concentration at which the peptide elicited 50% of the maximal Ca2+ flux (elicited by 10 μM ionomycin). BBC: backbone cyclic peptide.

In general all active linear peptides, regardless of whether they activated Hep-PK/PBAN-R or S/pl-PK/PBAN-R, exhibited dose–response curves, reaching peak activities within a range of 0.001 pM–10 pM (depending on the peptide), and then decreasing gradually to control levels. All active linear peptides were highly potent and exhibited EC50 values in the fM range (Table 2). It is important to note that because the level of activation in some of the linear and BBC peptides was lower than 50% the response selectivity is not always indicated by the EC50 values. No stimulation was obtained in any of the experiments, in the absence of a peptide ligand, and no response was obtained in S9 cells that were transfected with just a pIZ/V5-His vector that did not contain a PK/PBAN-R gene insert (data not shown).

Examination of the activities of the various peptides on Hep-PK/PBAN-R (at 1 pM) revealed that MT, LPK, β-SGNP, and the BBC peptides BBC-7 and BBC-8 did not significantly differ in their ability to activate the receptor compared with that of PBAN. The activities of the C-terminal-derived peptide PBAN 28–33 and the free-acid PBAN (PBAN-COOH) as well as DH and BBC-5 differed significantly from that of PBAN. In the case of S/pl-PK/PBAN-R only two peptides differed in their activity from PBAN: β-SGNP and PBAN-COOH. All other peptides showed similar activity to that of PBAN (Fig. 8).

**Discussion**

In the present study two cloned receptors (a H. petiti gera pheromone gland receptor Hep-PK/PBAN-R, which stimulates sex pheromone biosynthesis, and S. littoralis larval receptor S/pl-PK/PBAN-R, which mediates cuticular melanization in moth larvae) were stably expressed in S. frugiperda (S9) cells and their ability to respond to several native PK/PBAN peptides (β-SGNP, MT and LPK), and to linear and conformationally constrained backbone cyclic analogs derived from their sequences was monitored by measuring free intracellular Ca2+. The findings revealed that not all peptides exhibited the same potency: some peptides selectively activated one but not the other receptor; some were non-selective and activated both receptors at similar concentrations; and some activated both receptors but exhibited higher activity (either dose- or response-wise) toward one compared with the other. All active linear peptides were highly potent and exhibited EC50 values in the fM range (Table 2). BBC peptides (BBC-7 and BBC-8) stimulated the receptors at the aM range, indicating a very high affinity of these peptides toward the receptors (BBC-7 toward the Hep-PK/PBAN-R and BBC-8 toward both receptors), and hinting at the possibility that the PK/PBAN-R response may be enhanced by the formation of a GPCR signalosome, which is sensitive to aM concentrations of ligand, as reported for the mammalian Np relaxin [11].

The selectivity of both receptors toward another PK/PBAN-derived peptide (DH) has previously been demonstrated by us by means of a similar Ca2+ assay [12]. In that study we have shown that S/pl-PK/PBAN-R exhibited a high response to DH, with EC50 of 0.013 pM, whereas Hep-PK/PBAN-R was not activated by this peptide over a very wide concentration range, i.e., fM to μM. Both receptors responded to PBAN with EC50 values in the sub-pM range (0.15 and 0.12 pM, for Hep- and S/pl-PK/PBAN-R, respectively) [12].

The differential responses of the two receptors to β-SGNP, DH, and BBC-5 strengthen the general perception that Hep- and S/pl-PK/PBAN-R represent two different variants or two sub-classes of
Fig. 7. Dose–response of BBC-8-stimulated Ca\(^{2+}\) flux in (A) Hep-PK/PBAN-R-expressing and (B) Spl-PK/PBAN-R-expressing Sf9 cells. All experimental details are as described in Fig. 2. Results represent the means ± SEM of four independent experiments with each receptor, each of which included four replicates.

Fig. 8. Effects of various peptides on Ca\(^{2+}\) flux in Hep-PK/PBAN-R (HP-PBANR) and Spl-PK/PBAN-R (SL-PBANR)-expressing Sf9 cells as compared with PBAN. Data of the activity of DH have been published previously [11]. Values represent the mean ratio (expressed as a percentage) ± SEM between the maximal activity of each peptide and that of 1 pM PBAN. A pound sign (#) indicates significant differences in activity between PBAN and any tested peptide on the same receptor (either Hep-PK/PBAN-R or Spl-PK/PBAN-R).
the PK/PBAN-R family. This has been previously proposed by us in light of our use of homology modeling techniques that revealed significant structural differences at the N-termini, in the second extracellular loop (ECL), the third intracellular loop (ICL), and the C-terminus, and in light of a comparison between the electrostatic potentials calculated for the two receptor models, which revealed a large net charge difference in the extracellular regions and a smaller one at the C-terminus [12]. Since the electrostatic force is clearly the most important long-range attractive force, these differences in the regions known to be important for the identification and interaction with ligands might account for the differing affinities toward the two receptors obtained for the ligands tested in the present and the previous study.

An indication of possible differences between these two receptors came also from our previous in vivo studies with the conformationally constrained BBC PK/PBAN analogs. As indicated in the results BBC compounds exhibited receptor selectivity. Examination of the agonistic and antagonistic properties of BBC PK/PBAN analogs by means of in vivo pheromonomotropic and melanotropic assays, revealed the presence of selective agonists and antagonists toward each function which resulted, most likely, from differences in the binding pocket of the receptors that mediate these functions; differences that enabled binding of only some BBC peptide(s), thereby mediating one function but not the other.

Until now PK/PBAN-Rs have been cloned from the pheromone glands of adult female moths of several Lepidopteran species, from moth larvae and pupae, and also from D. melanogaster, R. prolixus and the mosquitoes, A. gambiae and A. aegypti. The receptors were expressed in various cell lines and their ability to bind and respond to different PK/PBAN ligands was tested by monitoring free intracellular Ca2+ [4,10,12,20,22]. The phylogenetic relationships between some of them have been recently evaluated [20]. Examination of the responses of these receptors to a variety of PK/PBAN ligands revealed several other receptors that showed selective responses, as indicated by their ability to induce Ca2+ flux. A pupal receptor from B. mori responded selectively to PK/PBAN peptides: it exhibited a high response only to Bom-DH but not to any of three other peptides – PBAN, β- and γ-SGP [13]. Two D. melanogaster receptors – Drm–CG8784 and Drm–CG8795 – selectively responded to Drm–β-SGP but not to Drm–DH [27]; and an Ang-R of the mosquito A. gambiae (termed Ang-PK-1) responded to Ang-DH but not to Ang-PBAN or Ang–β-SGP [21]. In all the above cases the receptor either did not respond to a given peptide ligand or elicited Ca2+ flux at concentrations three to four times as high as those required of the active peptides.

Examination, in our studies as well as in those of other laboratories, of the doses of PK/PBAN-derived peptides (PBAN, DH, LPK, β- and γ-SGP) used in stimulation of PK/PBAN-Rs from pheromone glands of Lepidopteran species revealed EC50 values in the nM range [9,16], and an activity range between 1 nM and 1 μM for Bom-R [13]. Drm–PK/PBAN-Rs and Aea–PK/PBAN-R were activated at a similar range [10,27]. Our present results with the pheromone gland Hep–PK/PBAN-R revealed five to six orders of magnitude higher sensitivity toward LPK and β-SGP (EC50 of 0.05 and 0.001 pM, respectively) and 4–6 orders of magnitude difference in their sensitivity toward PBAN [12]. Only one other pheromone gland receptor was tested for its response with Lom–MT-II; it revealed activity at 1 nM [16]; in the present study Hep–PK/PBAN-R was slightly more active, with peak activity at 0.1 pM.

In contrast to the well-studied sex pheromone PK/PBAN-Rs, to date only three other larval receptors have been cloned and characterized: from H. virescens larvae that were functionally inactive [16], from D. melanogaster [6], which responded to β-SGP and CAPA–DH at 400 and 50 nM, respectively, and from R. prolixus which responded to DH α- and β-SGP in the μM range [22]. Similarly to other studies, in the present study the Spl–PK/PBAN-R larval receptor did not respond to β-SGP at sub-nM range. In contrast to the lack of response toward β-SGP our S. littoralis larval receptor was several orders of magnitude more potent toward DH than what has been reported for the Rhp–PK/PBAN-Rs, with an EC50 of 0.013 pM [12] compared with 958 and 422 nM (for A and B variants, respectively) [22]. None of the larval receptors was tested for its response to LPK or MT.

The reason for the differences in EC50 values between our present findings and those of other studies is not fully understood, especially with regard to those between the H. zeu-R and our cloned H. peltigera receptor; H. zeu and H. peltigera are closely related moths and both receptors have been expressed in the same cell line. One possible explanation is that they represent different PK/PBAN receptor variants in the pheromone gland, and that each cloned receptor represents a different variant (as has been reported for several moth species by Lee et al. [18]). It also could be that the differences between the responses of the pheromone gland receptors resulted from use of differing experimental paradigms for their characterization, for example: different expression systems (stable or transient); use of different cell lines (insect Sf9 or CHO); use of modified receptors (native or enhanced green fluorescent protein (EGFP)-linked); use of modified ligands, i.e., Rhodamine-labeled PBAN; differing Ca2+ indicators; differing equipment sensitivity, etc. [8,14,16,19]. Support for the possible effect of the experimental set up on the receptors characteristics comes from our previous study, in which the cloned Spl–PK/PBAN-R was stably expressed in NIH3T3 cells and its ability to elicit Ca flux was monitored in the presence of PBAN [30]. The receptor responded to PBAN but the range of activity was much higher, at 0.1–1 μM, than the sub-pM concentrations obtained when the receptor was expressed in an Sf9 insect expression system [12]. Indeed, several previous studies have also indicated that the proper receptor ligand interaction is very much dependent upon the choice of the expression system and the mode of expression. Whatever the reason, the physiological significance of the present data calls for further exploration.

The differential responses to β-SGP and DH raise a few interesting issues and questions with regard to the mode of action of this family of peptides. The present findings indicate that although the C-terminally conserved sequence of the family (FXPRLa), which is shared by all PK/PBAN peptides and constitutes its active site and is important for its bioactivity, is not the only sequence that determines and contributes to ligand/receptor interactions, and that there are, most likely, additional residues that play a role in ligand binding.

Despite the intense studies of the interactions between PK/PBAN-Rs and the peptides of this family at the cellular level our understanding of the correlation between the cellular functional response and a specific in vivo bioactivity (sex pheromone biosynthesis in adult female moths and larval cuticular melanization) is still very limited. In light of the present results and of our previous study [12] we may speculate that PBAN, LPK and MT (which stimulated both receptors) are, most likely, involved in regulation of both functions, whereas β-SGP and DH stimulate only one function each – pheromone biosynthesis and melanization, respectively).

Molecular cloning of the PK/PBAN peptides revealed that in moths, PBAN is encoded by the pban-gene, which also encodes β-SGP and DH. DH (which contains the consensus sequence – WFGPLRa) is also encoded by the capa-gene and is termed CAPA-DH [4]. This raises the question of whether the selective response of the two receptors indicates responsiveness of Spl–PK/PBAN-R to both gene products – pban-gene-derived PBAN and DH and capa-gene-derived DH – and responsiveness of Hep–PK/PBAN-R to only the pban-gene products – PBAN and β-SGP. This issue needs to be further evaluated with regard to other peptides encoded by these two genes (α- and γ-SGP encoded by the pban-gene and two periviscerokinins – PVK-1 and PVK-2 – which are encoded by
the capa-gene and contain a PRLa sequence at their carboxy terminus; for peptide terminology please refer to [4]). This issue will also need to be re-evaluated once the Spodoptera and the Heliothisae capa-gene has been cloned, and the capa-gene-derived DH (CAPA-DH) amino acid sequences determined.

Another interesting issue in this connection relates to the fact that PBAN, β-SGP and DH are contained within the same pro-hormone encoded by the pban-gene. Thus, unless there are special cell-type differential processing mechanisms, such as those found for the capa-gene in D. melanogaster and B. mori [23,26], all three peptides must be produced and released in equimolar amounts by the neurosecretory cells. Thus, the inability of the DH to stimulate the Hep-PK/PBAN-R pheromone gland receptor and that of β-SGP to stimulate the Spl-PK/PBAN-R seems peculiar, especially in light of the fact that DH elicits sex pheromone biosynthesis in vivo in H. peltigera females – albeit at a 100-times higher dose than PBAN – and that β-SGP does stimulate cuticular melanization (Alstein et al., in preparation). This raises the possibility that DH may act on another receptor present in the pheromone gland, and β-SGP on another larval receptor. Indeed, recent studies have shown concurrent expression of multiple PK/PBAN pheromone gland receptor variants that arise from alternative splicing of the 3' coding region in several moth species (B. Mori, H. ze a, H. armigera, and P. separata) – variants that share a significant homology and differ from each other in an extended C-terminal sequence. Attempts to clone such receptors from the H. peltigera and S. littoralis moths are needed, in order to check whether there are additional variants in these two insects and to characterize their cellular functional properties in response to stimulation with different PK/PBAN ligands. The possible presence of two or more receptors for a single gene product suggests that several simultaneous physiological processes have to be coordinated or synchronized in order to elicit a given biological activity. Studies along these lines should be further pursued.

In summary: many attempts have been made to characterize the PK/PBAN receptors, in efforts to gain a better insight into the mode of action of this family of Nps and into the molecular mechanisms underlying the wide variety of functions mediated by them. The present findings on the functional differences between the two PK/PBAN-R (which mediate sex pheromone biosynthesis in adult female moths and larval cuticular melanization) represent strong and direct evidence for the presence of receptor sub-classes (or variants) and for the notion that different PK/PBAN in vivo functions are mediated by different PK/PBAN receptors. The findings also hint at the possibility that each function might be elicited by a different peptide or peptides. Cloning and stable expression in cell lines of the PK/PBAN-Rs may now pave the way to answering some of the key questions regarding the bioactivity of this family of Nps. Further research with these expressed receptors will aim to identify additional conformation aspects that are critical to the binding of pban- and capa-gene-derived peptides to the active site, and their activation, and also to explore the respective signaling pathways associated with the activation of the various receptors by different PK/PBAN ligands. Further characterization of these receptors and of their interactions with the various members of the PK/PBAN family peptides might advance our understanding of their multifunctional nature in moths and other insects, shed light on as yet unknown important processes mediated by the family, and help in exploring novel high-affinity receptor-specific antagonists that might serve as a basis for the development of new families of insect-control agents.

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