Biostable β-amino acid PK/PBAN analogs: Agonist and antagonist properties

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ABSTRACT

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family plays a significant role in a multifunctional array of important physiological processes in insects. PK/PBAN analogs incorporating β-amino acids were synthesized and evaluated in a pheromonotropic assay in Heliothis peltigera, a melanotropic assay in Spodoptera littoralis, a pupariation assay in Neobellieria bullata, and a hindgut contractile assay in Leucophaea maderae. Two analogs (PK-βA-1 and PK-βA-4) demonstrate greatly enhanced resistance to the peptidases neprilysin and angiotensin converting enzyme that are shown to degrade the natural peptides. Despite the changes to the PK core, analog PK-βA-4 represents a biostable, non-selective agonist in all four bioassays, essentially matching the potency of a natural PK in pupariation assay. Analog PK-βA-2 is a potent agonist in the melanotropic assay, demonstrating full efficacy at 1 pmol. In some cases, the structural changes imparted to the analogs modify the physiological responses. Analog PK-βA-3 is a non-selective agonist in all four bioassays. The analog PK-βA-1 shows greater selectivity than parent PK peptides; it is virtually inactive in the pupariation assay and represents a biostable antagonist in the pheromonotropic and melanotropic assays, without the significant agonism of the parent hexapeptide. These analogs provide new, and in some cases, biostable tools to endocrinologists studying similarities and differences in the mechanisms of the variety of PK/PBAN mediated physiological processes. They also may provide leads in the development of PK/PBAN-based, insect-specific pest management agents.

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1. Introduction

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family of peptides plays a multifunctional role in the physiology of insects. In 1986 the first member of the family, leucopyrokinin (LPK), was isolated from the cockroach Leucophaea maderae [14] with over 30 members of this peptide class identified thereafter. All family members share the common C-terminal pentapeptide FXPRL-amide (X = S, T, G or V) and include subfamilies such as pyrokinins, myotropins (MTs), PBAN, diapause hormone (DH), melanization and reddish coloration hormone (MRCH), pheromonotropin (PT), as well as pheromonotropic β and γ peptides derived from the cDNA of moths [3,29,30]. The PK/PBAN family has been shown to stimulate sex pheromone biosynthesis in moths [3,29–31], and mediate critical functions associated with feeding (gut contractions) [21,33], development (egg diapause, pupal diapause and pupariation) [15,22,23,25,28,35,36] and defense (melanin biosynthesis) [5,18] in a variety of insects. The peptides do not exhibit species specificity and experiments have shown that all of the functions listed above can be stimulated by more than one peptide [1,11,29,30]. The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides operate and agonists and antagonists, particularly selective ones, can shed light on this issue.

While PK/PBAN molecular messengers are both potent and specific, they are not suitably designed to be effective either as pest insect control agents and/or tools for insect neuroendocrinologists. Neuropeptides are rapidly degraded by peptidases in the hemolymph and tissues within insects and generally exhibit poor bioavailability [2,23,26,27]. The development of potent agonists and antagonists with enhanced biostability can overcome at least one of these limitations and can represent a key step in the development of pest management techniques based on neuropeptide analogs capable of disrupting critical life processes regulated by the PK/PBAN family.
# Biostable b-amino acid PK/PBAN analogs: Agonist and antagonist properties

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family plays a significant role in a multifunctional array of important physiological processes in insects. PK/PBAN analogs incorporating b-amino acids were synthesized and evaluated in a pheromonotropic assay in Heliothis peltigera, a melanotropic assay in Spodoptera littoralis, a pupariation assay in Neobellieria bullata, and a hindgut contractile assay in Leucophaea maderae. Two analogs (PK-bA-1 and PK-bA-4) demonstrate greatly enhanced resistance to the peptidases neprilysin and angiotensin converting enzyme that are shown to degrade the natural peptides. Despite the changes to the PK core, analog PK-bA-4 represents a biostable, non-selective agonist in all four bioassays, essentially matching the potency of a natural PK in pupariation assay. Analog PK-bA-1 shows greater selectivity than parent PK peptides; it is virtually inactive in the pupariation assay and represents a biostable antagonist in the pheromonotropic and melanotropic assays, without the significant agonism of the parent hexapeptide. These analogs provide new, and in some cases, biostable tools to endocrinologists studying similarities and differences in the mechanisms of the variety of PK/PBAN mediated physiological processes. They also may provide leads in the development of PK/PBAN-based insect-specific pest management agents.
The PKs are hydrolyzed by tissue-bound peptidases at a primary susceptibility site between the P and R residues within the C-terminal pentapeptide sequence that defines members of this family of neuropeptides [27]. Incorporation of β-amino acids can enhance resistance to peptidase attack and modify biological activity [7,16]. Indeed, this strategy has been successfully applied to the insect kinin neuropeptide family [34,37], leading to the identification of several potent selective and non-selective agonists with enhanced bioactivity characteristics. In this article we describe the synthesis of a number of analogs of the PK/PBAN C-terminal hexapeptide core in which the important residues Tyrβ, Pheβ, and Proβ are replaced with β3-aminooxy acid and/or their β2-homo-α-amino acid counterparts. All analogs were blocked at the N-terminus with an Ac group, which confers resistance to hydrolytic degradation by an additional class of peptidases, the aminopeptidases [13]. We have shown that the C-terminal PK hexapeptide analog is susceptible to degradation by the pure peptidases nepripisin (NEP) and angiotensin converting enzyme (ACE), whereas two of the β-amino acid analogs were found to demonstrate significantly enhanced resistance to these same enzymes.

The β-amino acid PK/PBAN analogs PK-βA-1 to -4 (structures listed below) were also tested for their ability to elicit and/or inhibit pheromone biosynthesis in the moth Heliotris peltigera and melanization in the Egyptian cotton leaf worm Neobellieria bullata and hindgut contraction in the cockroach L. maderae as examined as well.

2. Materials and methods

2.1. Insects

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. 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.

H. peltigera moths were reared on an artificial diet as described previously [10]. 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 described previously [10]. All females used in this study were 3.5 days old.

Larvae of the flesh fly, N. bullata were reared in batches of 200–300 specimens on beef liver in small open disposable packets made from aluminum foil as described [36]. Fully grown larvae that left the flesh were allowed to wander in dry sawdust until the first puparia appeared 36–40 h later. The batch was ready for collecting when red spiracle (RS) stage larvae, distinguished by precocious tanning of the cuticle in the region of hind spiracles (peritreme), appeared. For the bioassay early-RS larvae (2–3 h before puparization) were used, unless indicated otherwise. L. maderae cockroaches were kept in plastic containers at 30 °C with a light:dark regimen of 12:12. Food and water were provided ad libitum [14].

2.2. β-Amino acid PK/PBAN analog synthesis and purification

Four β-amino acid PK/PBAN analogs were synthesized:

- PK-βA-1: Ac-YFTβ3PmRLa (1461)
- PK-βA-2: Ac-YFTβ3PmOPTTRLa (1466)
- PK-βA-3: Ac-YFTβ3PmOPTTRLa (1465)
- PK-βA-4: Ac-[β3PmF]FTβ3PmRLa (1602)

Protected β-amino acids were purchased from Fluka (Buchs, Switzerland). PK/PBAN analogs were synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Applied Biosystems, Foster City, CA) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) under previously described conditions [37]. Crude products were purified on a Waters C18 Sep Pak cartridge and a Delta Pak C18 reverse-phase column (8 mm × 100 mm, 15 μm particle size and 100A pore size) on a Waters 600 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA) and Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: initial solvent consisting of 10% B was followed by the Waters linear program to 90% B over 25 min; flow rate, 2 ml/min. Delta Pak C18 retention times: PK-βA-1 (Ac-YFTβ3PmRLa): 8.5 min; PK-βA-2 (Ac-YFTβ3PmOPTTRLa): 11.0 min; PK-βA-3 (Ac-YFTβ3PmOPTTRLa): 9.0 min; PK-βA-4 (Ac-[β3PmF]FTβ3PmRLa): 7.5 min. The peptides were further purified on a Waters Protein Pak 1125 column (7.8 mm × 300 mm) (Milligen Corp., Milford, MA). Conditions: flow rate: 2.0 ml/min; Solvent A = 95% acetonitrile made to 0.01% TFA; Solvent B = 50% aqueous acetonitrile made to 0.01% TFA; 100% A isocratic for 4 min, then a linear program to 100% B over 80 min. WatPrO retention times: PK-βA-1 (Ac-YFTβ3PmOPTTRLa): 6.25 min; PK-βA-2 (Ac-YFTβ3PmOPTTRLa): 6.0 min; PK-βA-3 (Ac-YFTβ3PmOPTTRLa): 6.25 min; PK-βA-4 (Ac-[β3PmF]FTβ3PmRLa): 6.0 min. Amino acid analysis was carried out under previously reported conditions [37] and used to quantify the peptide and to confirm identity, leading to the following analyses: PK-βA-1 (Ac-YFTβ3PmOPTTRLa): F[1.0], L[1.0], R[1.0], T[0.9], Y[0.9]; PK-βA-2 (Ac-YFTβ3PmOPTTRLa): L[1.0], P[0.9], R[0.9], T[0.9], Y[0.9]; PK-βA-3 (Ac-YFTβ3PmOPTTRLa): L[1.0], P[0.9], R[0.9], T[0.9], Y[0.9]; PK-βA-4 (Ac-[β3PmF]FTβ3PmRLa): F[1.0], L[1.0], T[1.1], R[1.0]. The identity of the peptide analogs were confirmed via MALDI-TOF MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical Ltd., Manchester, UK) with the presence of the following molecular ions (M+H+): PK-βA-1 (Ac-YFTβ3PmRLa): 851.4 [calc MH+ = 851.9]; PK-βA-2 (Ac-YFTβ3PmOPTTRLa): 865.9 [calc MH+ = 866.0]; PK-βA-3 (Ac-[β3PmF]FTβ3PmRLa): 851.0 [calc MH+ = 851.0]; PK-βA-4 (Ac-[β3PmF]FTβ3PmRLa): 849.5 [calc MH+ = 849.1].

2.3. Synthesis of PBAN-33NH2, PT and LPK

Hez-PBAN [32] and Pseudaleia (Mythimna) separata PT (Pss-PT) [19] were synthesized on an ABI 433A automatic peptide synthesizer on Rink amide 4-methylbenzhydrylamine (MBHA) resin by means of the FastMoc™ chemistry as described previously [14]. Syntheses of L. maderae (Lem-LPK) [14] and PBAN fragment–analogs YFTTRLa and the acylated version (Ac-YFTTRLa, 1559) were carried out via 9-fluorenylmethoxycarbonyl (Fmoc) methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Advanced Chemtech, Louisville, KY) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) as described previously [27]. The purity of all peptides was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) [5] 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 hydrolysates.

2.4. Pheromonotropic bioassay

The pheromonotropic bioassay was performed with H. peltigera as described previously [4]. Stimulatory activity of PBAN, LPK, the β-amino acid PK/PBAN analogs and the LPK derived peptide 1559...
was determined by monitoring their ability to induce sex pheromone biosynthesis. Females injected with 1 pmol PBAN-33NH₂ served as a reference for stimulatory activity. Antagonistic activity was determined by monitoring the ability of the tested peptides to inhibit sex pheromone biosynthesis that was elicited by PBAN-33NH₂ at 1 pmol. Females injected with the elicitor served as a reference for maximal stimulation and those injected with 100 mM phosphate buffer served to determine the basal pheromone biosynthesis at photophase. The pheromone content in buffer-injected moths did not exceed 10 ng/female. The pheromone glands were excised 2 h post-injection and sex pheromone was extracted and quantified by capillary gas chromatography as described previously [4]. All experiments were performed with 8–10 females per treatment.

2.5. Melanotropic bioassay

The melanotropic bioassay was performed as described previously [6]. Melanotropic stimulatory activity of PBAN, LPK, the β-analogs or the LPK derived peptide 1559 was determined by evaluating their ability to induce cuticular melanization in larvae. Larvae injected with 5 pmol PBAN-33NH₂ served as a reference for stimulatory activity. Antagonistic activity was determined by monitoring the ability of the β-analogs (at 1 nmol), injected together with the elicitors PBAN-33NH₂, PT or LPK (at 5, 5 and 15 pmol, respectively), to inhibit cuticular melanization. Larvae injected with the elicitors at the indicated doses served as reference for maximal stimulation, and those injected with 50 mM HEPES, pH 7.6 served to determine the basal cuticular melanization of the ligated insects. Each experiment also involved analysis of the intensity of the melanized area in untreated and ligated larvae. The cuticular melanization was quantified as the ratio between the optical density and the scanned cuticular area (in millimeters) and was compared between control and experimental animals. All experiments were performed with 8–10 larvae per treatment. The only experiments taken into account were those in which the extent of melanization in buffer-injected larvae did not differ significantly from that of ligated animals, and did differ significantly from that of those injected with PBAN-33NH₂ (5 pmol).

2.6. Pupariation bioassay

The test was performed as described by Zd'arek et al. [36]. Briefly, the tested material was injected at doses of 0.5, 5, 50 and 500 pmol into flesh fly larvae (N. bullata) at the early-RS stage that previously had been immobilized by chilling on ice. Control larvae were injected with water only. After removal from the ice the injected larvae were kept at 25 °C in Petri dishes lined with dry filter paper, and the time of retraction (R), contraction (C) and tanning (T) was recorded. At the end of the RS stage the larva stops crawling and irreversibly retracts the first three front segments with the cephalopharyngeal apparatus (‘the mouth hooks’) (retraction—R); it then contracts longitudinally to become the barrel-shaped puparium (contraction—C) and its surface becomes barrel-shaped puparium (contraction—C) and its surface becomes

2.7. Myotropic bioassay

Hindguts of adult L maderae cockroaches were isolated from the central nervous system (CNS) and dissected [14], suspended in a 5 ml chamber, and prepared for recording as previously described [8]. Threshold concentrations were determined for each analog by adding a known quantity (dissolved in 0.5 ml of bioassay saline) [14] to the bioassay chamber containing the hindgut. The threshold concentration was defined as the minimum concentration of analog required to elicit an observable change in the frequency (50%) or amplitude (10%) of contractions within 1 min and sustained for 3 min. Threshold concentrations were obtained from measurements of three to five cockroach hindguts on consecutive days. A test for potential antagonist activity was conducted by introduction of LPK at a concentration of 3 × 10⁻³ M (15 pmol) in the quantity required to produce a half-maximal response on the hindgut, followed by a β-amino acid analog at a concentration of 5 × 10⁻⁶ M (25 nmol) to determine if it could inhibit the initial response.

2.8. Enzyme hydrolysis trials

2.8.1. Angiotensin converting enzyme trials

Drosophila ACE (M₀, 67,000) was purified from a soluble extract of adults as described elsewhere [9,17] and yielded enzyme that appeared as a single band by SDS-PAGE. PK/PBAN β-amino acid analogs (100 μM) were incubated at 35 °C with 25 ng ACE in 0.1 M HEPES buffer, pH 7 (total volume, 20 μl) at 30 min. The reaction was stopped by the addition of 5 μl TFA to a final concentration of 2.7% (v/v) and the volume was made up to 260 μl with 0.1% (v/v) TFA before HPLC analysis.

2.8.2. Neprilysin degradation trials

PK/PBAN β-amino acid analogs (100 μM) were incubated at 35 °C with 20 ng human recombinant neprilysin (a gift from Dr. A.J. Kenny, School of Biochemistry and Molecular Biology, University of Leeds) in 0.1 M HEPES buffer, pH 7 (total volume, 20 μl) at 35 °C for 30 min. The reaction was stopped by the addition of 5 μl TFA to a final concentration of 2.7% (v/v) and the volume was made up to 260 μl with 0.1% (v/v) TFA before HPLC analysis.

HPLC analysis of the fragments after ACE and NEP degradation was performed using a Jupiter 5 μ, column (C18, 250 mm in length × 4.5 mm, internal diameter; Phenomenex, Macclesfield, UK) and UV detection at 214 nm, and a linear gradient (6–50%) of acetonitrile in 0.1% TFA over 25 min at flow rate of 1 ml/min. Rates of hydrolysis were calculated from the percentage decline of the substrate, as measured by changes in peak height, and in comparison with a substrate standard treated under the same conditions, but without enzyme. Hydrolysis never exceeded 20% of the starting substrate concentration to ensure that the reaction was linear with time. Assays were performed in triplicate. More experimental detail is presented in a previous manuscript [17].

2.9. Statistical analysis

The results of the pheromontropic, melanotropic and hindgut contractile assays were subjected to one-way ANOVA. All data are presented as mean ± standard error mean. The significance of differences among means was evaluated with the Tukey–Kramer HSD (honestly significant difference) test at P < 0.05. The threshold concentration data for each active analog in the cockroach hindgut contractile assay were presented as (mean ± standard deviation) and were calculated using the student T test software (JMP version 5.1.2, ©2004, SAS Institute Inc., Cary, NC, USA).
3. Results

3.1. Pheromonotropic bioassay

The results of a dose–response evaluation of the four β-amino acid PK/PBAN analogs as agonists in the in vivo pheromonotropic assay in H. peltigera indicate that the analogs demonstrated marked differences in their activity. Substitution of the Proβ with a β3-αmino acid or the Pheα with a β2-homo-αmino acid (as in PK-βA-1 (1461) and PK-βA-2 (1466), respectively) resulted in a marked loss in activity compared to the parent acylated C-terminal hexapeptide Ac-YFTPRLα (1559) (Fig. 1). However, substitution of the Pheα with a β2-αmino acid (as in PK-βA-3, 1465) resulted in a highly potent agonist that exhibited activity even at 1 pmol, and substitution of both Pheα and Proβ with two β3-αmino acids (as in PK-βA-4, 1602) resulted in the most potent agonist which exhibited the highest activity at 1 pmol and at 1 nmol among all β substituted peptides.

Comparison of the activity at 1 pmol (Fig. 1) revealed that peptide PK-βA-4 is the most active one, likely due to enhanced biostability. The activity of this peptide (which stimulated pheromone biosynthesis to a level of 24 ± 10 ng, n = 10) was slightly higher than that of the parent peptide Ac-YFTPRLα (that stimulated pheromone biosynthesis to 11 ± 6 ng, n = 10) and equipotent with that of LPK at this dose (23 ± 12 ng pheromone, n = 10). At 10 pmol, the activity of PK-βA-4 was similar to that of the parent peptide Ac-YFTPRLα (61 ± 21 ng n = 10 and 60 ± 5 ng, n = 10, respectively) and to that of LPK (82 ± 14 ng, n = 10).

All peptides demonstrated high activities at 1 nmol (ranging from 68 to 126 ng) with PK-βA-4 being the highest amongst the β substituted peptides (68 ± 9 ng, 84 ± 6 ng, 80 ± 10 ng, and 118 ± 14 ng, n = 10 for PK-βA-1, PK-βA-2, PK-βA-3, and PK-βA-4, respectively, Fig. 1). Its activity was higher than that of all the other peptides and equipotent with that of the parent peptide Ac-YFTPRLα (118 ± 14 ng, n = 10 and 126 ± 15 ng, n = 10, respectively). Interestingly, the activities of both PK-βA-4 and the parent peptide 1559 at 1 nmol were higher than that of LPK (95 ± 15 ng, n = 10), although they did not differ significantly. The activity of all peptides, whether substituted or not, was significantly lower at 1 pmol than that of PBAN (Fig. 1).

The β-amino acid substituted peptides that were weak agonists (PK-βA-1 and PK-βA-2) were tested for pheromonotropic antagonistic activity at different doses. The data in Fig. 2 show that peptide PK-βA-1 exhibited a significant antagonistic activity at 100 pmol (a dose at which the peptide was devoid of agonistic activity). Peptide PK-βA-2 was inactive at all tested doses. The other peptides (PK-βA-3, PK-βA-4 and the control parent acylated peptide 1559) were tested for their antagonistic activity at 100 pmol and 1 nmol. The data revealed that all three peptides were devoid of any inhibitory activity at both doses.

3.2. Melanotropic bioassay

Unlike in the pheromonotropic assay substitution of Pheα and Proβ by one or two β3-αmino acids or a β2-homo-αmino acid did not result in marked differences between the peptides in the melanotropic activity. All peptides were active to some extent with three out of the four substituted peptides (PK-βA-2, PK-βA-3, and PK-βA-4) being highly potent even at 1 pmol (Fig. 3). No dose dependency was observed with any of the tested peptides and the response seemed to be of an ‘all or none’ nature. The parent acylated C-terminal hexapeptide Ac-YFTPRLα exhibited the highest activity matching the potency and efficacy of the 33-residue natural peptide PBAN (Fig. 3) at all tested doses.

Remarkably, analog PK-βA-2 and PK-βA-4 elicited melanotropic activity at a dose of 1 pmol, which did not differ significantly from that of PBAN at the 5 pmol dose and from that of the unmodified parent hexapeptide (Fig. 3). These analogs therefore matched the potency of the unmodified parent peptide and PBAN, despite the structural modification to the Pheα position and were significantly much more active than LPK at the same dose. As in the pheromonotropic assay, analogs PK-βA-2, PK-βA-3 and PK-βA-4...
were highly active at 1 nmol exhibiting an activity that did not differ significantly from that of PBAN but was significantly lower than that of the native peptide (1559) (Fig. 3). Weak melanotropic activity was detected for analog PK-bA-1 at all tested doses (Fig. 3).

An evaluation of the β-amino acid PK/PBAN analogs as antagonists in the in vivo melanotropic assay in S. littoralis against several natural elicitors is presented in Table 1. When evaluated against PBAN, PK-bA-3 is completely devoid of any inhibitory activity. Similar results were obtained with the two other natural elicitors PT and LPK (Table 1).

Each of the other analogs PK-bA-1, PK-bA-2, and PK-bA-4 at a 1 nmol dose inhibited the melanotropic activity of PBAN by 100%, 84%, and 65%, respectively, and all statistically significant. The acylated parent hexapeptide Ac-YFTPRLa inhibited PBAN melanotropic activity by a statistically significant 53%. PK-bA-4 showed a statistically significant inhibition of PBAN (73%) at a dose of 100 pmol as well (data not shown). The other two analogs PK-bA-1 and PK-bA-2 did not show any significant inhibition at doses of 100, 10 or 1 pmol (data not shown). Similar inhibitory activities were obtained with those peptides when Pss-PT was used as an elicitor where PK-bA-1, PK-bA-2, and PK-bA-4 inhibited PT elicited melanin formation by 99%, 99%, and 89%, respectively (Table 1). The peptides fail to inhibit LPK in the melanotropic assay (Table 1), with the exception of 10 pmol PK-bA-1 that inhibited LPK by 57% (data not shown).
Table 1
Summary of antagonistic melanotropic activity of β-amino acid PK/PBAN analogs on different elicitors.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Antagonistic activity a (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Elicitors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBAN1-33NH2 (5 pmol)</td>
</tr>
<tr>
<td>β-AA PK/PBAN analogs (1 nmol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK-βA-1 (1461)</td>
<td>Ac-YFT[β3P]RLa</td>
<td>100 ± 1 (n = 10) *</td>
</tr>
<tr>
<td>PK-βA-2 (1466)</td>
<td>Ac-Y[β2homotr]TPRLa</td>
<td>84 ± 11 (n = 10) *</td>
</tr>
<tr>
<td>PK-βA-3 (1465)</td>
<td>Ac-Y[β3F]TPRLa</td>
<td>1 ± 3 (n = 10)</td>
</tr>
<tr>
<td>PK-βA-4 (1602)</td>
<td>Ac-[β3F][F][β3P]RLa</td>
<td>65 ± 17 (n = 8)</td>
</tr>
<tr>
<td>Control peptide (1 nmol)</td>
<td>Ac-YFTPRLa</td>
<td>53 ± 14 (n = 10) *</td>
</tr>
</tbody>
</table>

* Antagonistic activity (e.g., inhibition) is expressed as 100 minus the ratio (as percentage) between the melanin formation elicited by each elicitor in the presence and absence of the tested peptides.

** An activity that differs significantly (at P < 0.05) from that obtained by the elicitor itself.

Table 2
Pupariation acceleration (Neobellieria bullata) and hindgut contractile (Leucophaea maderae) agonist activity of β-amino acid PK/PBAN analogs.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pupariation (pmol)</td>
</tr>
<tr>
<td>LPK</td>
<td>pETSFIPRLa</td>
<td>0.3 [28]</td>
</tr>
<tr>
<td>PK-βA-3 (1465)</td>
<td>Ac-[β3F]TPRLa</td>
<td>0.5</td>
</tr>
<tr>
<td>PK-βA-2 (1466)</td>
<td>Ac-[β2homotr]TPRLa</td>
<td>5</td>
</tr>
<tr>
<td>PK-βA-1 (1461)</td>
<td>Ac-YFT[β3P]RLa</td>
<td>500</td>
</tr>
<tr>
<td>PK-βA-4 (1602)</td>
<td>Ac-[β3F][F][β3P]RLa</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

* The threshold concentrations listed in the hindgut contraction column of the table are equivalent to 5, 35, 50, 75, and 50 pmol in the experimental chamber for LPK, PK-βA-3, PK-βA-2, PK-βA-1, and PK-βA-4, respectively.

3.3. Pupariation and myotropic bioassays

The results of an evaluation of the β-amino acid PK/PBAN analogs in the larval pupariation assay of N. bullata and isolated hindgut contraction assay of the cockroach L. maderae are summarized in Table 2. The PK Lem-PK is native to the L. maderae cockroach and also the first PK sequence used to establish the core region, as in analogs PK-βA-1 and PK-βA-4 has stabilized them to both ACE and NEP peptidases. No hydrolysis of PK-βA-1 could be detected for either, whereas the hydrolysis of PK-βA-4 has been greatly reduced to 4% (ACE) and 9% (NEP) of the rates for the unmodified parent PK peptide YFTPRLa (Table 3).

4. Discussion

Despite the incorporation of an additional methylene group (–CH₂–) within the backbone of the C-terminal pentapeptide core region of PK/PBAN peptides, single or double replacement of amino acids with their β-amino acid counterparts led, in selected cases, to significant retention of biological activity in several insect PK/PBAN bioassays. In some cases, the structural modification of the β-amino acid analogs led to a modification of the activity profile of the parent acylated, PK/PBAN C-terminal hexapeptide. Notably, the substitution of a β⁻¹-Pro⁸ for the Pro⁸ in the middle of the core region, as in analogs PK-βA-1 and PK-βA-4, stabilized the resulting analogs to pure peptidases that degrade, and therefore inactivate, the natural PK/PBAN peptides. No hydrolysis of analog PK-βA-1 could be detected, even when extended incubation times were employed, when exposed to ACE and NEP peptidases, and the hydrolysis of PK-βA-4 was greatly reduced to 4% and 9%.

3.4. Enzyme hydrolysis trials

Two of the analogs (PK-βA-1: Ac-YFT[β3P]RLa and PK-βA-4: Ac-[β3F][F][β3P]RLa) were selected for enzyme hydrolysis trials with pure NEP and ACE because they featured a β-amino acid replacement adjacent to the primary tissue-bound peptidase site located in the middle of the critical C-terminal PK/PBAN pentapeptide active-core sequence [27]. The other two analogs were not protected in this critical active-core region, and were therefore not considered likely to be ‘biostable’. The results are summarized in Table 3. The placement of β-amino acids in PK-βA-1 and PK-βA-4 has stabilized them to both ACE and NEP peptidases. No hydrolysis of PK-βA-1 could be detected for either, whereas the hydrolysis of PK-βA-4 has been greatly reduced to 4% (ACE) and 9% (NEP) of the rates for the unmodified parent PK peptide YFTPRLa (Table 3).
respectively, of the rates observed for the unmodified parent PK/PBAN hexapeptide. Notably, the introduction of a second β3-Phe as a replacement for the Phe\(^2\) in the N-terminal region of the core sequence actually somewhat reduced, though did not reverse, the protection from peptidase hydrolysis afforded by the β3-Pro\(^4\) in the middle of the core. This result suggests that the active site of the enzymes are extended enough that substitutions some distance away from the susceptible region can influence hydrolysis rates, in this case a negative influence. The other two analogs did not involve incorporation of β-amino acids adjacent to the primary tissue-bound peptidase hydrolysis site \[27\] of the core sequence, and were therefore not evaluated against the two peptidases.

The double β-amino acid analog PK-βA-4 demonstrated non-selective agonism in all of the bioassays—that is pupariation, pheromonotropic, melanotropic, and hindgut contractile assays. Notably, PK-βA-4 virtually matched the activity of the natural PK Lem-PK in the flesh fly pupariation bioassay, and was equipotent or more active than the parent peptide and Lem-PK at most tested doses in the pheromonotropic assay. It therefore represents a potent, biostable puperation and pheromonotropic agonist in flesh fly larvae and \(H.\) \textit{peltigera} female moths. Several previous examples exist of biostable analogs of insect neuropeptides that feature less potent intrinsic biological activity over natural peptides, but nonetheless, demonstrate enhanced \textit{in vivo} activity \[12,20,24\]. Like the parent acylated, PK hexapeptide, this double-β analog elicited a mixture of both agonist and antagonist responses in the melanotropic assay of \(S.\) \textit{littoralis} and pure agonistic pheromonotropic activity in \(H.\) \textit{peltigera} (Table 4).

The analog PK-βA-3 is a non-selective agonist in all four of the PK/PBAN bioassays. Indeed, it matches the potency of the natural PK Lem-PK in the flesh fly pupariation bioassay. Unlike the acylated parent hexapeptide, this analog is a pure pheromonotropic and melanotropic agonist, as it fails to demonstrate statistically significant inhibition of any of the natural elicitors used in the \(H.\) \textit{peltigera} or \(S.\) \textit{littoralis} assays (Table 4). The analog PK-βA-2 demonstrates a potent agonist response in the \(S.\) \textit{littoralis} melanotropic bioassays, even demonstrating full efficacy at a dose of 1 pmol, as is observed for the unmodified acylated, parent PK hexapeptide (Fig. 3 and Table 4). The related analog PK-βA-2 features a β3-homo-Phe as a surrogate for Phe rather than the β3-Phe in PK-βA-3. In comparison with Phe, additional methylene groups are located between the alpha carbon and the amino group, as well as between the alpha carbon and the side chain group in β3-homo-Phe. Only one additional methylene group is found in β3-Phe, located between the alpha carbon and the carboxyl group. As a result of the more extensive structural change, analog PK-βA-2 features a complex activity profile in comparison with PK-βA-3 in the four bioassays. Like PK-βA-3, PK-βA-2 demonstrates an agonist response in the hindgut contractile, melanotropic and pupariation assays, although it is an order of magnitude less potent than PK-βA-3 in the latter bioassay. However, PK-βA-2 fails to demonstrate an agonist response in the pheromonotropic assay. And whereas PK-βA-3 is a pure agonist in the melanotropic assay, PK-βA-2 demonstrates a significant antagonist response against the elicitors PBAN and Pss-PT.

The structural modification in analog PK-βA-1 that led to strong resistance to peptidase attack by ACE and NEP also led to modifications of its agonist profile in the various assay systems, increasing its selectivity over the acylated parent hexapeptide and/or natural PK/PBANs. This analog shows only very weak activity in the pupariation assay, placing it at the margin between activity and inactivity. Unlike the parent hexapeptide, analog PK-βA-1 demonstrates no statistically significant agonist activity in the melanotropic assay (Fig. 2) and at a wide range of doses in the pheromonotropic activity with the exception of 1 nmol where its activity reached 68% of that of PBAN (Fig. 1). It does, however, demonstrate antagonist activity against elicitors Pss-PT and PBAN in the melanotropic assay and against PBAN in the pheromonotropic assay (Fig. 2). It therefore represents a non-selective biostable antagonist (Table 4).

The β-amino acid analogs described in this study provide new, and in some cases, biostable tools to endocrinologists studying similarities and differences in the mechanisms of the spectrum of PK/PBAN-regulated physiological functions in insects. They also may provide leads in the development of novel insect-specific, environmentally favorable pest management agents capable of disrupting PK/PBAN-regulated systems.

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