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Bioavailability of β-amino acid and C-terminally derived PK/PBAN analogs☆

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ABSTRACT

The ability of linear β-amino acid substituted peptides (PK-βA-1: Ac-YFT[β^3P]RLa; PK-βA-2: Ac-Y[β^3homoF]TPRLa; PK-βA-3: Ac-Y[β^3F]TPRLa; PK-βA-4: Ac-[β^3F]FT[β^3P]RLa) and unsubstituted analogs (Ac-YFTPRLa and YFTPRLa) of the pyrokinin(PK)/pheromone biosynthesis-activating neuropeptide (PBAN) family to penetrate the insect cuticle and exert biological activity (i.e., stimulate sex pheromone biosynthesis), was tested by topical application on *Heliothis peltigera* moths. The present results clearly indicate that small linear synthetic peptides can penetrate the cuticle very efficiently by contact application and activate their target organ. The time responses of the peptides applied in DDW and DMSO were tested and the activities of topically applied and injected peptides were compared. The results clearly indicate that PK-βA-4 and PK-βA-3 exhibited high bioavailability (ability to penetrate through the cuticle and exertion of bioactivity) with the latter showing longer persistence in both solvents than any other analog in the study; indicative that incorporation of a β-amino acid at the Phe^2 position can enhance longevity in topical PK/PBAN analogs. PK-βA-4 was significantly more active in DMSO than in DDW, and significantly more active than the parent peptide LPK in DMSO. PK-βA-1 and PK-βA-2 exhibited negligible activity. Interestingly, Ac-YFTPRLa was highly potent in both solvents; its activity in DDW did not differ from that of PK-βA-4 and PK-βA-3, and was higher than that of LPK. Even the unacylated peptide YFTPRLa was active in both solvents, at a similar level to LPK. Topically applied PK-βA-4 and Ac-YFTPRLa exhibited significantly higher activity than the injected peptides. PK-βA-3 and YFTPRLa were equally potent in both routes of administration.

1. Introduction

Insect neuropeptides (Nps) are prime targets in studies of the physiology and behavior of insects, and in the search for novel insecticides, since they regulate many physiological and behavioral processes during insect development, reproduction and senescence. Understanding their modes of action may shed light on and reveal basic endocrine mechanisms of general interest, and use of their blockers (antagonists) or super-agonists may disrupt and interfere with normal growth, development and behavior of insects and, therefore, could lead to receptor-selective, insect-specific insecticides or control agents. The chemical nature of Nps enables their use as the basis for the design of a generic group of non-toxic insecticides, through an approach similar to that applied to human Nps as a novel direction in the pharmaceutical industry.

Despite their great potential, not a single Np-based agonist or antagonist insect control agent has been made commercially available up to now, and there are no applications of Np-based compounds as pest-control agents. There are many reasons for this. First and foremost, no methodology has yet emerged for converting an endogenous agonist into a super-agonist or antagonist, and then into an insecticide or insect control agent. Second, even when such agonists or antagonists were identified, their practical application as insect control agents would be discouraged by the general perception that Nps have poor metabolic stability and low bioavailability, partly because of difficulty in crossing membrane barriers and, especially, the apolar lipid insect cuticle. Thus, conversion of an insect Np to an insecticide prototype requires, in addition to highly potent agonistic or antagonistic properties and high selectivity toward insect pests, availability of cost-effective compounds with high metabolic stability and, most important, high bioavailability, i.e., ability to penetrate the hydrophobic insect cuticle and reach their target organ. To obtain molecules with the above characteristics there is a need to modify them so that they fulfill the above requirements.

In the past few years, we have devised a few novel integrated approaches to the design of highly potent, metabolically stable,

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and bioavailable antagonists. One approach, designated insect Np antagonist insecticide, INAI, was based on substitution of L-amino acids with D-Phe followed by backbone cyclization [14]. Introduction of D-Phe into peptides has proven effective in conversion of agonists to antagonists ([40] and references therein). Backbone cyclization forms molecules with: enhanced metabolic stability, which results from lower susceptibility to degrading enzymes; high selectivity, which results from conformational space restriction, and allows the peptides to mediate one function and excludes it from other functions; increased biological activity, which is facilitated by the much slower equilibrium between conformations; and improved bioavailability, which results from reduced polarity. Thus, backbone cyclization has proven effective in generating a few potent, stable and selective Np analogs [12,14,15]. This strategy was applied to the pyrokinin (PK)/pheromone biosynthesis-activating neuropeptide (PBAN) family of insect Nps, and led to the generation of several highly potent, conformationally constrained, selective, bioavailable and metabolically stable antagonists [2,4,5,9,11,39,40]. Other approaches, based on generation of linear amphiphilic, peptidase-resistant pseudopeptides of the PK/PBAN family, were also examined and led to generation of highly potent and stable analogs, capable of penetrating the insect cuticle [1,21,23,27–29,31,36,37].

Successful implementation of the above approaches provided important information on the structural requirements of highly potent, selective, biostable and bioavailable agonists and antagonists; information that is essential for the rational design of insect control agent prototypes. However, these compounds by themselves cannot serve as insecticides, because their synthesis requires long and costly processes that, in most cases, lead to expensive compounds. This presents a real obstacle to their practical application, especially since insect control agents must be cost-effective. Thus, there is a need to seek for simple compounds onto which the above structural requirements can be imposed. In a previous study we demonstrated, for the first time, that native, unmodified, linear peptides of various lengths belonging to the PK/PBAN family (PBAN, *Leucophaea maderae* PK termed LPK, pheromonotropin, PT, and myotropin, MT) were highly bioavailable, i.e., could penetrate the insect cuticle, and could reach and activate their target organ when topically applied, in double distilled water (DDW) or dimethylsulfoxide (DMSO), to the female moth abdomen [16]. These results raised the question of whether synthetic short (six amino acid) peptides, which are more prone to proteolytic degradation, were also bioavailable and, if so, what are the properties that would enable them to penetrate the cuticle and reach the target organ. Proof of bioavailability of such peptides can dramatically simplify the strategies that should be considered in development of rationally designed, environmentally friendly and biodegradable, control agents, based on Np agonists or antagonists, and can add significantly to neuroendocrine studies of moths.

Recently, we have designed four PK/PBAN linear analogs incorporating β-amino acids, derived from the C-terminal hexapeptide core (YFTPRLa) of the PK/PBAN family, in which the important residues Tyr[1], Phe[2], and Pro[4] were replaced with β^3-amino acid and/or their β^2-homo-amino acid counterparts, and the N-terminus was blocked with an acyl (Ac) group [21]. The peptides were designed to confer resistance to hydrolytic degradation by endopeptidases such as neprilysine (formerly termed enkephalinase), dipeptidyl peptidases such as angiotensin-converting enzyme (ACE), and aminopeptidases [10,19], and, indeed some of them were found to be highly potent and metabolically resistant [21]. In light of our recent findings of the high bioavailability of linear peptides, we investigated, in the present study, the ability of these peptides to penetrate the insect cuticle and to exert bioactivity, e.g., stimulation of sex pheromone biosynthesis, when applied topically onto *Heliothis peltigera* moths, in DDW or DMSO. The tested peptides comprised four synthetic short β-amino acid substituted peptides – PK-βA-1: Ac-YFT[β^3P]RLa (1461); PK-βA-2: Ac-Y[β^2homoF]TPRLa (1466); PK-βA-3: Ac-Y[β^3F]TPRLa (1465); PK-βA-4: Ac-[β^3F]FT[β^3P]RLa (1602) – and two analogs—(YFTPRLa, 1323), and the acylated peptide (Ac-YFTPRLa, 1559), all derived from the parent peptide LPK. The present results clearly indicate that small linear synthetic peptides can penetrate the cuticle very efficiently following contact application in aqueous solutions or organic solvents, and can reach their target organ and activate it. These findings also highlight the important residues in the molecules which confer on them the necessary properties.

2. Materials and methods

2.1. Insects

H. peltigera moths were reared on an artificial diet as described previously [13]. 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 in the wild by means of pheromone traps, as described previously [13]. All females used in this study were 3.5–4.5 days old.

2.2. Peptide synthesis

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

Four β-amino acid PK/PBAN analogs were synthesized [21]:

PK-βA-1: Ac-YFT[β^3P]RLa (1461);
PK-βA-2: Ac-Y[β^2homoF]TPRLa (1466);
PK-βA-3: Ac-Y[β^3F]TPRLa (1465);
PK-βA-4: Ac-[β^3F]FT[β^3P]RLa (1602).

Protected β-amino acids were purchased from Fluka (Buchs, Switzerland). PK/PBAN analogs were synthesized by means of 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) and purified under previously described conditions [43].

2.2.2. Synthesis of LPK and LPK derived peptides (1323 and 1559)

Syntheses of *L. maderae* (Lem-LPK) [17], the LPK fragment-analogs YFTPRLa (1323) and its acylated version (Ac-YFTPRLa, 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 [30]. The purity of all peptides was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) as previously described [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.

2.3. Pheromonotropic bioassay

2.3.1. Topical application

The cuticular scales on the ventral surface of the abdomen of 3.5- to 4.5-day-old *H. peltigera* females at photophase were gently removed by rubbing the abdomen with a cotton bud. Peptides were dissolved in DDW or DMSO (BDH, England), acetonitrile (HPLC, Supra gradient, Bio Lab, Jerusalem, Israel) or absolute ethanol (AR) (Bio Lab, Jerusalem, Israel) and 1 μl of the peptide

solution was applied to the surface of the cuticle. To ensure absorption of the applied peptides, the moths were immobilized, ventral side up, by clamping their wings with smooth-jawed alligator clips. Once the drop was absorbed, the clips were removed and the moths were transferred to screen cages supplied with 10% sugar solution. Females on which 1 nmol LPK was applied served as a reference for stimulatory activity and as a positive control, and those that received 1 μl of solvent (DDW, DMSO, acetonitrile or ethanol without peptides) were used for determination of the basal level of pheromone biosynthesis at photophase (negative control). All experiments were performed with a minimum of seven females per treatment in at least three independent experiments.

2.3.2. Peptide injection

The pheromonotropic bioassay was performed with *H. peltigera* as described previously [6]. Females injected with 100 mM phosphate buffer, pH 7.4 served as controls, to determine the basal level of pheromone biosynthesis at photophase. All experiments were performed with a minimum of 10 females per treatment in at least three independent experiments.

All peptides, whether injected or topically applied, were tested for their stimulatory activity, i.e., their ability to induce sex pheromone biosynthesis.

2.3.3. Pheromone gland excision and extraction

Pheromone glands of topically applied or injected females were excised at several different times post-treatment, and sex pheromone was extracted and quantified by capillary gas chromatography as described previously [6]. The pheromone content in control females did not exceed 6 ng/gland in either topical application or injection experiments.

2.4. Statistical analysis

All results were subjected to one-way ANOVA. The significance of differences among means was evaluated with the Tukey–Kramer honestly significant difference (HSD) test at $P < 0.05$. The data are presented as means ± S.E.M.

3. Results

3.1. Time responses of topically applied β-peptides in DDW

Examination of the bioavailability, i.e., ability to penetrate the insect cuticle and exhibit bioactivity, of four β-substituted peptides, applied topically at a dose of 1 nmol, indicated that some peptides were highly potent when applied in DDW for the indicated times, and were capable of stimulating sex pheromone biosynthesis in *H. peltigera* female moths. However, marked differences could be noticed among their activities (Fig. 1 and Table 1). PK-βA-3 (1465), in which the Phe^2 was substituted with a β^3-amino acid, was highly potent, eliciting a maximal activity of 187 ng pheromone when applied in DDW for 3 h. PK-βA-4 (1602), in which both Tyr^1 and Pro^4 were substituted with two β^3-amino acids, was the most potent of all the β-substituted peptides; it stimulated pheromone production to a maximal level of 210 ng at 3 h post-application. PK-βA-1 (1461) and PK-βA-2 (1466), in which the Pro^4 was substituted with a β^3-amino acid or the Phe^2 was substituted with a β^2-homo-amino acid, respectively, were inactive, i.e., they did not stimulate production of sex pheromone, when applied topically in DDW at a dose of 1 nmol for 1 or 3 h. Interestingly, the most potent of all tested peptides was the acylated peptide (Ac-YFTPRLa, 1559), which elevated pheromone production to 238 ng in 3 h. The non-acylated peptide (YFTPRLa, 1323) was also active, although less potent than the acylated analog, and its application resulted in synthesis of only 104 ng pheromone, 3 h post-application, similar to that of LPK, which stimulated sex pheromone biosynthesis to a level of 132 ng. The pheromone content in females which were topically applied just with 1 μl of DDW (without peptide) was negligible and ranged from 0 to 3 ng.

Examination of the time-course activity of the β-substituted peptides revealed that all active peptides, regardless of their potency, showed maximal activity 3 h post-application (Fig. 1). The activity of PK-βA-3 (1465) persisted longest (up to 8 h), and its activity at this time point was higher than that of the natural peptide LPK, although the levels of pheromone did not differ

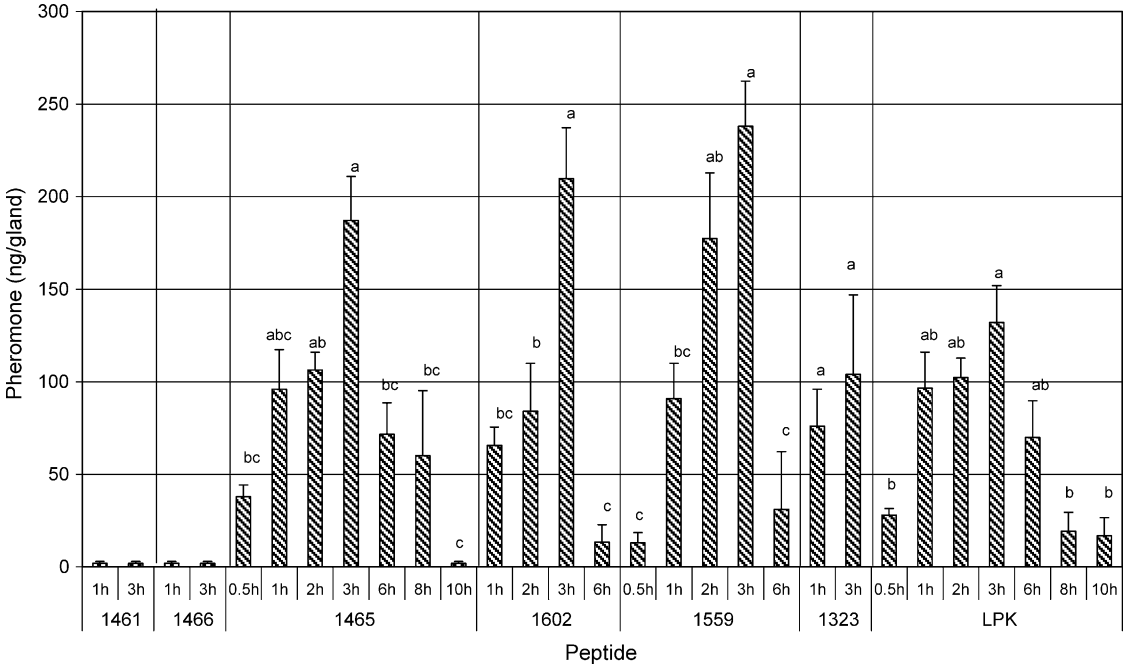

Fig. 1. Time responses of topically applied β peptides (PK-βA-1, 1461; PK-βA-2, 1466; PK-βA-3, 1465; PK-βA-4, 1602), the acylated (Ac-YFTPRLa, 1559), the non-acylated (YFTPRLa, 1323) C-terminal hexapeptide, and LPK. Peptides were applied in DDW at a dose of 1 nmol. Values represent means ± S.E.M. of a minimum of seven samples. Statistical analysis compared the activities of each peptide (separately) at the tested time points. Bars with the same letter do not differ significantly (at $P < 0.05$).

Table 1
Amounts of sex pheromone produced in response to topical application of β-substituted peptides, acylated and non-acylated C-terminal hexapeptides, and LPK.

Peptide	DDW	DMSO	DDW	DMSO
1 nmol	1 h		3 h	
PK-βA-1 (1461)	1 ± 1^{b} ($n = 8$)	33 ± 20^{c} ($n = 10$)	1 ± 1^{c} ($n = 8$)	2 ± 1^{d} ($n = 10$)
PK-βA-2 (1466)	2 ± 1^{b} ($n = 8$)	$36 \pm 7^{bc*}$ ($n = 8$)	1 ± 1^{c} ($n = 8$)	$19 \pm 8^{d*}$ ($n = 10$)
PK-βA-3 (1465)	96 ± 21^{a} ($n = 8$)	129 ± 55^{a} ($n = 7$)	187 ± 24^{ab} ($n = 7$)	238 ± 21^{ab} ($n = 7$)
PK-βA-4 (1602)	66 ± 10^{ab} ($n = 10$)	$99 \pm 9^{abc*}$ ($n = 8$)	210 ± 28^{ab} ($n = 8$)	$325 \pm 39^{a*}$ ($n = 7$)
1559	91 ± 19^{a} ($n = 19$)	121 ± 10^{a} ($n = 17$)	$238 \pm 24^{a*}$ ($n = 17$)	151 ± 27^{bc} ($n = 16$)
1323	76 ± 20^{ab} ($n = 10$)	120 ± 12^{ab} ($n = 10$)	104 ± 43^{bc} ($n = 10$)	107 ± 35^{cd} ($n = 10$)
LPK	97 ± 19^{a} ($n = 8$)	108 ± 7^{abc} ($n = 8$)	132 ± 20^{b} ($n = 16$)	165 ± 16^{bc} ($n = 18$)

Peptides were applied at a dose of 1 nmol in DDW or DMSO at the indicated times. Values represent means ± S.E.M. Statistical analysis compared the activities of the various peptides at each time point in each solvent separately (indicated by letters). Values with the same letter do not differ significantly (at $P < 0.05$). Statistical analysis also compared the activity obtained in DDW with that in DMSO for each peptide.
* Significant differences (at $P < 0.05$).

significantly. The activity of the other peptides (PK-βA-4 and 1559) persisted up to 6 h post-application (Fig. 1).

3.2. Time responses of topically applied β-peptides in DMSO

The β-substituted peptides were also active in DMSO (Fig. 2 and Table 1). Peptide PK-βA-4 (1602), was again the most active among this group (and among all of the tested peptides) and exhibited the highest activity, stimulating pheromone production to a level of 325 ng after 3 h. Peptide PK-βA-3 (1465) was slightly less active, and its topical application resulted in the formation of 238 ng of pheromone. Peptides PK-βA-1 (1461) and PK-βA-2 (1466), which were inactive when applied in DDW, showed some activity when applied in DMSO (33 and 36 ng, respectively, 1 h post-application, Table 1), and the peptides 1559 and 1323 exhibited moderate activity of about 151 and 107 ng, respectively, after 3 h. The activity of peptide 1559 did not differ significantly from that of LPK; that of 1323 was slightly lower but also not significantly different from that of the parent native peptide (Table 1). The pheromone content in females which were topically applied just with 1 μl of DMSO (without peptide) was negligible and ranged from 0 to 2 ng.

Contrary to the results obtained when the peptides were applied in DDW, where activity maximized at 3 h post-application, the time dependency when they were applied in DMSO was less significant, and no marked differences were observed between 1 and 3 h post-application, with the exception of PK-βA-4 (1602), which showed significantly higher activity at 3 h than at the other tested time points (Fig. 2). Similarly to the data obtained in DDW, the activity of PK-βA-3 (1465) persisted longer (6 h) than that of the most active analog PK-βA-4 (1602), which was active only for 3 h.

Comparison of the activities of 1-nmol doses of the β-peptides PK-βA-3 (1465) and PK-βA-4 (1602) and of the acylated and non-acylated analogs (1559 and 1323) with that of 1 nmol of the parent peptide, LPK revealed that at the time of their maximal activity, i.e., 3 h post-application, only the acylated analog (1559) exhibited significantly higher activity than LPK when applied in DDW, and only that of PK-βA-4 (1602) was higher than LPK when applied in DMSO (Table 1). The activity of the other active peptides did not differ significantly from that of LPK when applied in either DDW or DMSO (Table 1).

Comparisons among the activities of the active peptides, at 1 nmol, in DDW and in DMSO, at 1 and 3 h post-application revealed that the activity of PK-βA-4 (1602) was significantly higher in DMSO than in DDW at both time points, and that of 1559 was higher in DDW (Table 1). PK-βA-1 (1461) and PK-βA-2 (1466),

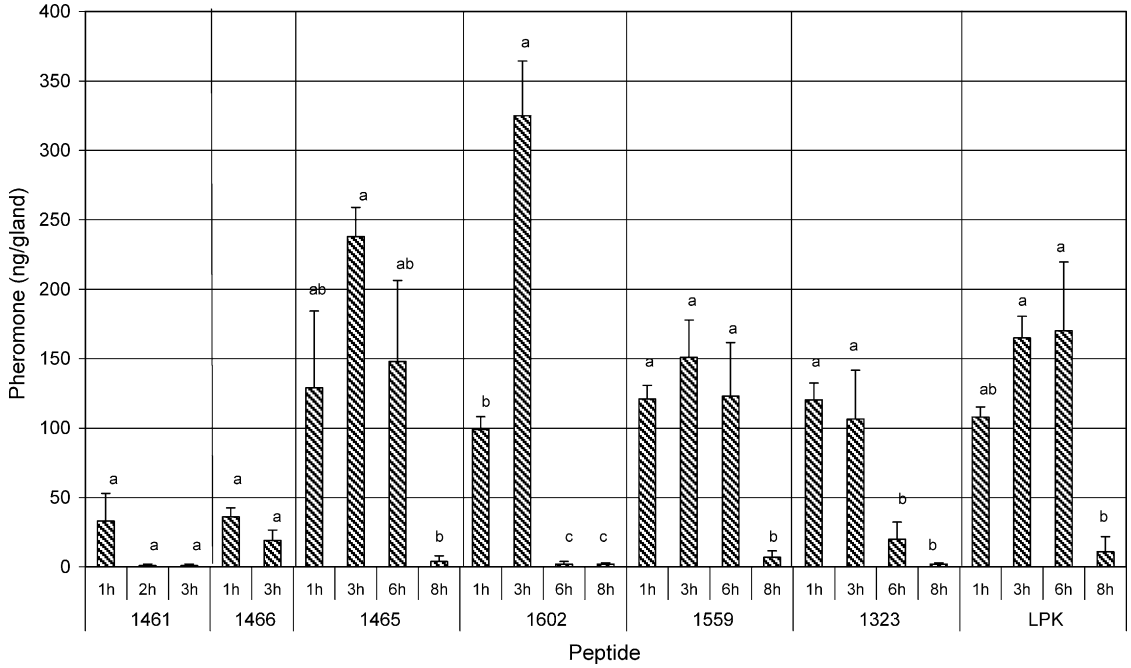

Fig. 2. Time responses of topically applied β peptides (PK-βA-1, 1461; PK-βA-2, 1466; PK-βA-3, 1465; PK-βA-4, 1602), the acylated (Ac-YFTPRLa, 1559), the non-acylated (YFTPRLa, 1323) C-terminal hexapeptide, and LPK. Peptides were applied in DMSO at a dose of 1 nmol. Values represent means ± S.E.M. of a minimum of seven samples. Statistical analysis compared the activities of each peptide (separately) at the tested time points. Bars with the same letter do not differ significantly (at $P < 0.05$).

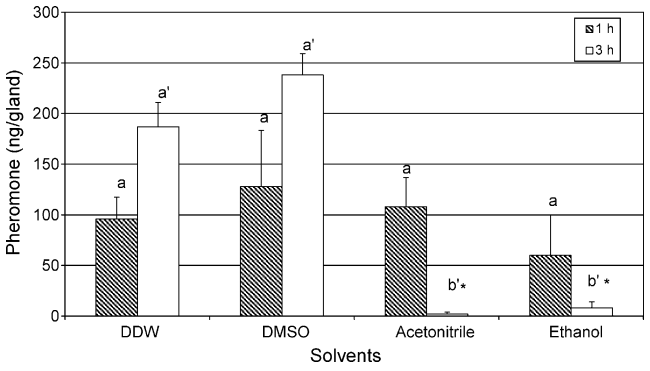

Fig. 3. Topical application of PK-βA-3 (1465) in several different solvents. The peptide was applied at a dose of 1 nmol and pheromone content was detected 1 and 3 h post-application. Values represent means ± S.E.M. of seven or eight samples. Statistical analysis compared the activities of the peptide in the different solvents at 1 and 3 h separately (indicated by letters). Bars with the same letter do not differ significantly. Statistical analysis also compared the activities of the peptide at 1 and 3 h, in each solvent separately. An asterisk (*) indicates a significant difference at $P < 0.05$.

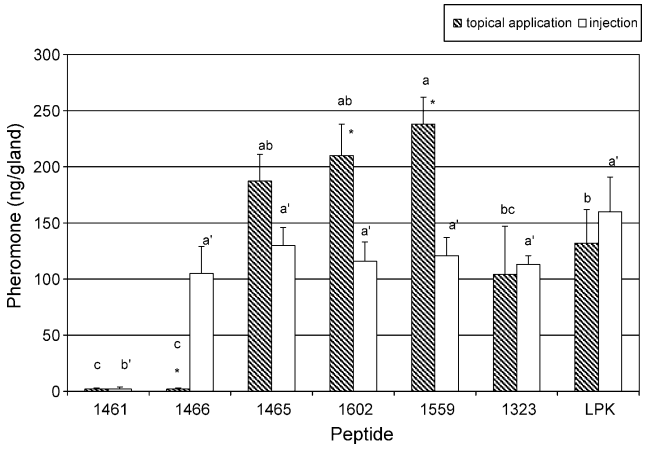

Fig. 4. Comparison between the activities of topically applied and injected peptides at a dose of 1 nmol. Peptides were topically applied or injected in an aqueous solvent, and glands were excised 3 h post-treatment. Values represent means ± S.E.M. of a minimum of eight samples. Statistical analysis compared the activities of the topically applied or injected peptides separately (indicated by letters). Statistical analysis also compared between the activities elicited by topical application and by injection for each peptide. An asterisk (*) indicates significant differences (at $P < 0.05$).

which exhibited no activity in DDW, were weakly active in DMSO, with 1466 being significantly more active than 1461 at 1 h post-application (Table 1). The activities of PK-βA-3 (1465), 1323 and LPK did not differ in the two solvents (Table 1).

3.3. Topical application of PK-βA-3 in different solvents

Examination of the activity of PK-βA-3 (1465) in acetonitrile and in ethanol revealed that its activity in these solvents at 1 h post-application did not differ significantly from that obtained in DDW or DMSO: 108 and 60 ng in acetonitrile and ethanol, respectively, compared with 96 and 129 ng in DDW and DMSO, respectively. The activity in the first two solvents, however, had short persistence, and no pheromone could be detected 3 h post-application (Fig. 3). Topically applied solvents did not stimulate any biosynthesis of pheromone.

3.4. Comparison between topically applied and injected peptides

The ability of the topically applied peptides, at a dose of 1 nmol, to stimulate sex pheromone biosynthesis was compared with that of the injected peptide. As indicated in Fig. 4, the activity of the topically applied β-peptide PK-βA-4 (1602) and that of the acylated peptide (1559) were significantly higher than those of the injected peptides: 210 and 238 ng pheromone, respectively, elicited by topical application, compared with 116 and 121 ng, respectively, by injection. Activities of topically applied and injected PK-βA-3 (1465) did not differ significantly (resulting in synthesis of 187 and 130 ng pheromone, respectively), similarly to the results obtained with the non-acylated peptide 1323 (104 and 113 ng, respectively) and LPK (132 and 160 ng, respectively) (Fig. 4). PK-βA-2 (1466) which exhibited no activity when applied topically stimulated pheromone biosynthesis to a level of 105 ng when injected. PK-βA-1 (1461) was inactive in both application methods (Fig. 4).

4. Discussion

The ability of linear β-amino acid modified peptides (PK-βA-1: Ac-YFT[β^3P]RLa; PK-βA-2: Ac-Y[β^2homoF]TPRLa; PK-βA-3: Ac-Y[β^3F]TPRLa; PK-βA-4: Ac-[β^3F]FT[β^3P]RLa), and two analogs (Ac-YFTPRLa and YFTPRLa) of the PK/PBAN family to exhibit bioavailability, i.e., to penetrate the insect cuticle and exert bioactivity, namely, stimulate sex pheromone biosynthesis, was tested by their topical application on adult female *H. peltigera* abdomens. The tested peptides were derived from a modification of the C-terminal hexapeptide core of the PK/PBAN family (FXPRLa, X = S, T, G or V), which is a ubiquitous multifunctional family that plays major physiological roles in regulating a wide range of developmental processes in insects: pupariation [32]; diapause [18,24,35,38,41,42]; cuticular melanization [8,20]; feeding, i.e., gut muscle contraction [22,34]; and mating behavior, i.e., sex pheromone production [6,33].

The β-substituted peptides were synthesized within the framework of a general effort to design improved PK/PBAN analogs, i.e., compounds with higher metabolic stability than the native peptides of the family [21]. The present series of β-substituted peptides was designed specifically to confer resistance to hydrolytic degradation by endopeptidases such as neprilysine (formerly termed enkephalinase), dipeptidyl peptidases such as angiotensin-converting enzyme (ACE), and aminopeptidases [10,19]. In a previous study we have shown that substitution of a β^3-Pro^4 for the Pro^4 in the middle of the core region stabilized the resulting peptides – PK-βA-1 (1461) and PK-βA-4 (1602) – against neprilysine and ACE, and that despite the incorporation of an additional methylene group (–CH_2–) within the backbone of the C-terminal pentapeptide core region of PK/PBAN peptides, single and/or double replacement of amino acids with their β-amino acid counterparts led, as in the case of analogs PK-βA-4 (1602) and PK-βA-3 (1465), to a significant retention of biological activity in several insect "PK/PBAN bioassays" including induction of pheromone biosynthesis in *H. peltigera* females [21]. All of the above studies were carried out by injection of the peptides into the insect.

In another recent study we have shown that the linear unmodified natural PK/PBAN elicitors PBAN, PT, LPK and MT exhibited high bioavailability, i.e., ability to penetrate the cuticle and exert bioactivity, namely sex pheromone biosynthesis, in *H. peltigera* moths. These results were obtained with topical application of 1-nmol doses, in either an aqueous solution or in DMSO [16]. In light of the finding that linear peptides were bioavailable, the bioavailability of the β-substituted peptides was examined, in order to test whether short synthetic peptides could transmigrate through the cuticle and exert bioactivity, and to examine how the above modifications affected bioavailability. In the present study we examined the bioavailability of the β-peptides when applied topically in DDW or in DMSO, assessed the

time response in the two solvents, and compared the activities of topically applied with those of injected peptides. An N-terminally acylated peptide and an unacylated peptide with the same primary sequence based on natural α-amino acids, designated 1559 and 1323, respectively, were examined in parallel to assess the contribution of the β-amino acids to the bioavailability.

The results clearly indicated that two β-substituted peptides – PK-βA-4 (1602) and PK-βA-3 (1465) – were highly potent when applied topically, either in DDW or in DMSO (Figs. 1 and 2). PK-βA-4 was significantly more active in DMSO than in DDW, and significantly more active than the parent peptide LPK when applied in DMSO for 3 h (Table 1). The other two β-peptides – PK-βA-1 (1461) and PK-βA-2 (1466) – exhibited no activity when applied in DDW (Fig. 1) and very low, negligible activity when applied in DMSO (Fig. 2). The difference in the activities of PK-βA-4 (1602) and PK-βA-3 (1465) in the respective solvents may be attributed to differences in their hydrophilic properties. The presence of an additional β-amino acid, containing an additional methylene group would be expected to increase the hydrophobic character of this analog. The persistence of the activity of both peptides in DDW indicates that they are very stable with PK-βA-3 (1465) being more stable than PK-βA-4 (1602) in both solvents. This may indicate the possibility that substitution of Phe^2 in 1465 with β^3F (in PK-βA-3) protects the peptide better against neprilysin or similar endopeptidases than the substitution of Tyr^1 with β^3F (in PK-βA-4), and that Phe^2 in the peptide is more susceptible to degradation by neprilysin than is Pro^4. Comparison of the time dependence patterns following application in DDW and DMSO revealed similar onset in the two solvents, and more persistent activity followed application in DDW than in DMSO (Figs. 1 and 2). The times to maximal activity were similar, at 3 h, in both solvents, hinting that accessibility to the target is similar for both β-peptides. The faster decline in the activities of PK-βA-4 (1602) and PK-βA-3 (1465) in DMSO than in DDW may have resulted from faster clearance of the peptide from the hemolymph in the presence of DMSO. Alternatively, the DMSO solvent may have hastened penetration of the analogs over water, thereby leading to a greater depletion of the supply of analog and decreasing time-release properties. A similar phenomenon was found with PBAN, which showed shorter persistence when topically applied in DMSO than in DDW [16].

The β-substituted analogs that exhibited activity when administered by topical application – PK-βA-4 (1602) and PK-βA-3 (1465) – were also the most active upon injection (for either 2 or 3 h; [21] and Fig. 4). PK-βA-2 (1466) was less active than PK-βA-4 (1602) or PK-βA-3 (1465) when injected for 2 h [21] and exhibited the same activity as these two peptides at 3 h (Fig. 4). Peptide (1466) was the only one that showed differing patterns when injected or applied topically, indicating that the substitution of Phe^2 with β^2homoF in PK-βA-2 drastically diminished the ability of the peptide to penetrate the cuticle when applied in DDW; this peptide did show marginal activity when applied topically in DMSO. PK-βA-1 (1461), administered by either route, was inactive at 3 h post-treatment. Thus, the "order of potency" of the β-substituted analogs was the same with both routes of administration for most β-substituted peptides, with the exception of PK-βA-2 (1466). Interestingly, the acylated LPK analog Ac-YFTPRLa (1559) was highly potent in both DDW and DMSO, and its activity in DDW did not differ from those of the active β-substituted peptides PK-βA-4 (1602) and PK-βA-3 (1465). The activity of 1559 lasted 6 h post-application in both solvents, and was significantly higher than that of the parent peptide, LPK when applied for 3 h in DDW (Figs. 1 and 2 and Table 1); it was significantly more active in DDW than in DMSO, most likely because its hydrophobicity is lower than that of PK-βA-4 (1602) because of the absence of β-amino acids from its sequence. Even the unacylated short linear peptide YFTPRLa (1323) was active in both solvents, and its activity did not differ significantly from that of LPK under all tested conditions (Table 1). The pheromonotropic activities of peptides 1559 and 1323, administered by injection, revealed that the acylated peptide 1559 was highly active and its activity did not differ significantly from those of PK-βA-4 (1602) and PK-βA-3 (1465) [21]. The unacylated peptide 1323 was slightly less active than 1559 (Altstein et al., unpublished). Thus, as with the β-substituted analogs, the "order of potency" of these peptides was the same for both routes of administration.

Another interesting aspect of the present findings relates to the long persistence (up to 6 h post-application) of the activity of acylated linear peptide 1559. This indicates that the peptide was very stable when applied in either DDW or DMSO (Figs. 1 and 2). The persistence resulted, most likely, from the introduction of the acyl group at the N-terminus of the molecule, which protects it against aminopeptidases. This notion is strengthened by the finding that the unacylated peptide, 1323 exhibited much lower and short-lived activity (Fig. 1). Addition of β-amino acids to the sequence, which acquired the active peptides with high resistance against proteolytic degradation by either neprilysin (PK-βA-3 and PK-βA-4) or ACE (PK-βA-4) did not improve the activity in comparison to that obtained with 1559, but resulted, in the case of PK-βA-3, in high persistence of up to 8 h, similar to that of PBAN [16]. This finding raises a possibility that the conformation created by the β-substitution did not enhance penetration through the cuticle compared with that of 1559, but extended the duration of the activity, because of its higher metabolic stability against endopeptidases such as neprilysin.

Comparison between the bioactivities of topically applied and injected peptides, at 1 nmol for 3 h, revealed that PK-βA-4 (1602) and the acylated peptide Ac-YFTPRLa (1559) exhibited significantly higher activities when topically applied than when injected (Fig. 4). PK-βA-3 (1465) and YFTPRLa (1332) were equally potent in both routes of administration, similar to LPK. PK-βA-2 (1466), which showed no activity when applied topically, was active when injected. Comparison of the activity of the topically applied peptides with the activity of those injected for 2 h [21] revealed a similar pattern of activity (data not shown). Since the peptides tested in the present study exhibited similar activities upon injection (Fig. 4) the differences in their bioavailability can be attributed to differences in their ability to transmigrate the cuticle and in their metabolic stability. The present data indicate that the conformations of the acylated peptide 1559 and of PK-βA-3 (1465) and PK-βA-4 (1602) give them the highest penetrability. Since the β-substituted peptides are more stable against proteolytic degradation, it could have been anticipated that their activity would be significantly higher than that of the acylated analog 1559. PK-βA-4 (1602) was significantly more active than 1559 when topically applied in DMSO (although less persistent), but the two were essentially equivalent in water. It is possible that the topically applied peptides either are not subjected to proteolysis in the hemolymph, or that their penetration through the cuticle is less than that of 1559 but their overall activity is boosted by their higher metabolic stability. This issue should be further investigated.

The lower activity obtained with the injected peptides than with the topically applied ones was quite unexpected in light of the fact the injected peptides are introduced in close proximity to the target organ, whereas the topically applied ones have to penetrate the cuticle, to be transported to the pheromone gland and then to exert their activity. One possible explanation for the high activity of the topically applied peptides is that injection imposes stress on the insect, which may impair sex pheromone biosynthesis. Another possible explanation is that the injected peptides are degraded by soluble or membrane-bound peptidases released from the damaged tissue, so that lower doses reach the target area.

Lower doses – 1–100 pmol – of topically applied PK-βA-3 (1465) and PK-βA-4 (1602) were also active although their activity was significantly lower than that of similar doses of the injected peptides (Altstein et al., unpublished). It is possible that, following their introduction into the insect, the topically applied peptides became more diluted in the hemolymph than the injected ones, which were applied in close proximity to the target organ. It should be noted, however, that, so far, for most of the tested peptides only DDW and DMSO were used as solvents for topical application, with the exception of PK-βA-3 (1465) that was also tested in acetonitrile and ethanol (Fig. 4). It could very well be that the use of other solvents at a wider range of time points might improve bioavailability at these doses.

Comparison between the bioavailability of the β-substituted peptides and the control acylated and unacylated peptides with that of the PK/PBAN natural (i.e., unmodified) elicitors PBAN, PT, LPK and MT (applied at a dose of 1 nmol for 3 h in DDW [16]) revealed that the acylated peptide 1559 and two of the β-peptides – PK-βA-3 (1465) and PK-βA-4 (1602) – exhibited bioavailability that did not differ significantly from that of PBAN, as indicated by the ability of 1559, PK-βA-3, PK-βA-4 and PBAN to stimulate sex pheromone biosynthesis to levels of 238, 187, 210 and 266 ng, respectively. The bioavailability of the unacylated control peptide, 1323, was lower than that of PBAN but similar to those of PT, LPK and MT, as indicated by the formation of 104, 132, 132 and 126 ng, respectively, of pheromone. Previous studies have indicated that PBAN was the most potent pheromonotropic elicitor in *H. peltigera*, as indicated by its ability to stimulate the highest levels of pheromone production and by its persistently high activity, both when injected and when topically applied. PT, LPK, MT or peptides based on the C-terminal sequence of the PK/PBAN family, e.g., TFSPRLa, were either less potent or less persistent [3,5,6,16]. The high potency of PBAN is not surprising in view of the fact that it is a native elicitor that evolved, most likely, to be highly potent and metabolically stable. Its high stability may result from an intrinsic or an acquired biostable conformation that could be imparted to the peptide either by the beta turn in the signature sequence (FSPRLa) of the PK/PBAN family [25,26], or as a result of its binding to carrier/scavenger proteins in the hemolymph during its transportation from the subesophageal ganglion (SOG) to the pheromone gland. Furthermore, PBAN is a big molecule (33 amino acids) that, even if degraded might still exhibit activity if the degradation occurred in the N-terminal part of the molecule, which was found to be unimportant for bioactivity [6]. Short peptides, like those that were tested in the present study, are much more susceptible than longer ones to proteolytic degradation [7] and cannot acquire complex conformations, because of their lower ability to form tertiary structures. In light of the above, our finding on the equivalent potency of 1559—a short, synthetic peptide of only six amino acids, protected only by an N-terminal acyl group with that of PBAN is quite remarkable. Even more interesting is the finding that LPK, which is highly homologous in its sequence to 1559 and is also protected at its N-terminus by a pGlu amino acid, exhibited lower bioavailability than 1559 when applied topically. These data indicate that bioavailability is determined not only by protection against proteolytic degradation but that it involves additional factors, most likely conformational, that determine cuticular transmigration. Since the ability of the three peptides, 1559, PBAN and LPK to stimulate sex pheromone biosynthesis when injected at a dose of 1 nmol for 3 h did not differ significantly – resulting in formation of 121, 179 and 160 ng pheromone, respectively – the present data on the high potency of 1559 clearly indicate that this peptide has the necessary conformational properties (as do analogs PK-βA-3 and PK-βA-4).

In summary, the present study yielded important new information on the bioavailability of linear peptides stabilized against proteolytic digestion in moths. The data clearly indicate that linear small synthetic peptides, in which the N- and C-termini are blocked and in which one or two amino acids in their sequence are substituted with β^2 or β^3 amino acids, can penetrate the cuticle following contact application in either aqueous or organic solvents, and can reach their target organ and activate it. This study is a continuation of our previous study on the bioavailability of PBAN, PT, LPK and MT, which revealed that linear unmodified peptides of various lengths can cross the cuticle and exert bioactivity [16]. Furthermore, it proves that the general notion that linear peptides of various lengths have low to negligible bioavailability via the insect cuticle may not always be accurate.

The present study reveals that protection of just the N-terminus (as in peptide 1559) is sufficient to acquire the peptide with high persistence and high potency, similar to that of the full-length most active elicitor PBAN, indicating the major role played by aminopeptidases in the degradation of active peptides in the hemolymph. These findings also indicate that substitution of α- with β-amino acid at various positions in the peptide molecule does not have a major effect on bioavailability, and in some cases even reduces the ability of peptides to transmigrate through the cuticle. An important exception is substitution of the Phe^2 with a β^3-Phe, which imbued the peptide with longer persistence, a desirable characteristic for pest management, and which indicates the participation of endopeptidases such as neprylisin in peptide degradation. ACE has, most likely, a minor role in PK/PBAN peptide degradation *in vivo*. Further studies on PK/PBAN analogs incorporating a β^3-Phe (as in PK-βA-3) and the persistence of their topical activity profiles are warranted.

Despite the relatively simple properties that imbue peptides with high bioavailability, cuticular transmigration of peptides and the time responses of their activities seem to present a more complex issue and to depend on combinations of a variety of properties and factors. Further studies on the peptide properties that lead to high cuticular penetration, persistence in the hemolymph and time-release profile should be undertaken, and their findings should be taken into consideration, together with previous findings on the hydrophobic and amphiphilic properties of such compounds [1,29–31,36,37], in the future design of bioavailable and bioactive compounds.

The results of the present study represent a significant addition to the approaches used in moth neuroendocrine studies: use of the noninvasive application route in such studies eliminates the stress and injury caused to the insect by injection—factors that could significantly affect the *in vivo* measurement of any physiological parameter. These results also add important information relevant to the strategies that should be considered in development of environmentally friendly, biodegradable, rationally designed control agents based on Np agonists or antagonists, which have been studied in our laboratory for many years (for review see [9]), and may lead to dramatic simplification of these strategies.

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