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Bioavailability of insect neuropeptides: The PK/PBAN family as a case study☆

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ABSTRACT

The ability of unmodified linear peptides to penetrate the insect cuticle and exert bioactivity (e.g., stimulation of sex pheromone biosynthesis) was tested by topical application onto *Heliothis peltigera* moths of four insect neuropeptides (Nps) of the pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family: *Helicoverpa zea* PBAN (Hez-PBAN), *Pseudaletia* (*Mythimna*) *separata* pheromonotropin (PT), *Leucophaea maderae* PK (LPK) and *Locusta migratoria* myotropin (Lom-MT-II). The time kinetic of the peptides applied in double distilled water (DDW) or dimethylsulfoxide (DMSO) was tested and the activities of topically applied and injected peptides were compared. The results clearly indicated that all four peptides were highly potent but with differing activities in the two solvents: PBAN was most active in water, and PT in DMSO. The activity of PBAN in DDW lasted up to 8 h post-application and its activity in this solvent showed a faster onset and a longer persistence than in DMSO. LPK and MT differed less in their kinetics between the two solvents. Topically applied PBAN at 1 nmol exhibited an equivalent or even significantly higher potency than the injected peptide at several different times post-treatment. Similar results were obtained with topically applied and injected LPK. The present results add important information on the bioavailability of unmodified linear peptides in moths, clearly indicate that linear hydrophilic peptides can penetrate the cuticle by contact application in aqueous solutions and in organic solvents very efficiently, reach their target organ and activate it.

1. Introduction

In the past few years, several major advances in research on insect neuropeptides (Nps) have enhanced our familiarity with their structures and functions. Many insect Nps have been identified, the basic principles of their action, e.g., biosynthesis, processing, release, transport, activation of the target cell, and degradation, have been discovered, and their roles in the physiology of organisms have been identified by means of genome sequencing, peptidomics, gene micro-arrays, receptor characterization, and targeted gene interference, all applied in association with physiological, electrophysiological and behavioral analyses. To date, about 30–40 genes encoding Np precursors have been predicted in several insect species, and a total of over 150 insect Nps have been characterized, the great majority of which are insect-specific. For review see [11,19,24,25,49].

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 the use of their blockers (antagonists) may disrupt and interfere with the normal growth, development and behavior of insects and, therefore could yield receptor-selective, insect-specific insecticides or control agents. The chemical nature of Nps enables them to be used as the basis for the design of a generic group of non-toxic insecticides similar to the approach that has been applied to human Nps as a novel direction in the drug industry. The major roles Nps play in the physiology of organisms, and their high potential for practical applications, have stimulated active interest in Np studies in general, and in those of insects in particular [3,4,10].

Although a vast amount of knowledge on insect Nps and their receptors has been accumulated in recent years, and in spite of the great potential of Np antagonists as insecticides, not a single Np-based antagonist insect control agent has been made commercially available up to now, and the application of Np antagonists as pest-control compounds does not exist. There are many reasons for this. First and foremost, no methodology has yet emerged for the conversion of an agonist to an antagonist and subsequently, into an insecticide or insect-control agent. Second, even when such

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Table 1
Amino acid sequence of the PK/PBAN peptides used in this study.

Code name	Insect species	Amino acid sequence	Reference
Hez-PBAN	*Helicoverpa zea*	LSDDMPATPADQEMYRQDPEQIDSRTKY**FSPRL**-$\mathbf{NH_2}$	[53]
Pss-PT	*Pseudaletia separata*	KLSYDDKVFENVE**FTPRL**-$\mathbf{NH_2}$	[32]
Lem-PK	*Leucophaea maderae*	pETS**FTPRL**-$\mathbf{NH_2}$	[27]
Lom-MT-II	*Locusta migratoria*	EGD**FTPRL**-$\mathbf{NH_2}$	[55]

Bold letters indicate conserved amino acid sequences of the PK/PBAN family. PBAN: pheromone biosynthesis activating neuropeptide; PT: pheromonotropin; PK: pyrokinin; MT: myotropin.

antagonists have been made, the general notion that Nps have a poor metabolic stability and their low bioavailability, which stems partly from their difficulty in crossing membrane barriers and, especially, the apolar lipid insect cuticle, would hinder their practical application as insect-control agents.

The very same problems have severely limited the development of therapeutic drugs based on human Nps, therefore, recent studies in medicinal chemistry have aimed to develop strategies to overcome these problems. These strategies included rational design and synthesis of chemically modified peptides [2], peptoids [57], backbone cyclic peptides [30], pseudopeptides [2] or peptidomimetic ligands [18,22,23,34], development of prodrugs [50], employment of delivery systems based on peptides linked covalently or non-covalently to cell penetrating peptides [14,31] or multifunctional polymers [15], and encapsulation of peptides in micelles and microemulsions [48]. These studies have led to the development of agonists and antagonists with reduced susceptibility to proteolysis, and enhanced bioavailability. These new approaches have improved the likelihood of obtaining useful drugs that are structurally related to the parent peptides, and have led to the development of a few peptide-based drugs for several human diseases [12,26,28,51]. Notwithstanding this progress, the technology has not yet been optimized, and most of the non-peptide ligands for Np receptors were identified by random screening of large chemical libraries, and were then chemically adapted to impart the desired selective and pharmacokinetic properties. There is still very limited information available on the conversion of an agonist to an antagonist, and new approaches are still being sought, to the generation of such antagonists and to the modification of native peptides to create stable and bioavailable peptidomimetic compounds with the desired features.

In the past few years, we have devised a novel integrated approach, designated **I**nsect **N**p **A**ntagonist **I**nsecticide, INAI – based on rational design – which overcomes some of the limitations associated with the application of Nps as insect control agents, and paves the way toward the development of Np antagonists. The method was tested with the pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family, and has led to the generation of a few highly potent, conformationally constrained, selective and metabolically stable antagonists [4–6,13,63,64]. Other approaches for generation of peptidase-resistant compounds that can penetrate the insect cuticle, and that were based on design of amphiphilic pseudopeptides, have also been introduced [1,35,38,42–44,46,59,60].

The successful implementation of the above approaches provided important information on the structural requirements of highly potent, selective, biostable and bioavailable agonists and antagonists, which is essential for the rational design of insect control agent prototypes. However, these compounds by themselves cannot serves as insecticides because their synthesis requires long and costly processes which, in most cases lead to costly compounds. This represents a real obstacle to their practical application, especially since insect control agents must be cost effective. Thus, the need to seek for simple compounds, onto which the above structural requirements can be implemented, does exists.

In this study we demonstrate, for the first time, that unmodified, linear peptides of various lengths are highly bioavailable, and can penetrate the insect cuticle and reach and activate their target organ. The study investigated four insect Nps of the PK/PBAN family: Hez-PBAN, Pss-PT, LPK and Lom-MT-II (Table 1. For nomenclature of peptides see [52]) which are known, among other functions, to be elicitors of sex pheromone biosynthesis in adult female *Heliothis peltigera* moths [4,10,54]. The peptides, in double distilled water (DDW) or dimethylsulfoxide (DMSO), were topically applied to the abdomens of the female moths, and their ability to stimulate sex pheromone biosynthesis was tested at several times post-application. The information presented in this paper forms a significant addition to the approaches used in moth neuroendocrinology studies in general, and to the strategies that should be considered in development of environmentally friendly, biodegradable, rationally designed pest control agents, based on peptide agonists or antagonists.

2. Materials and methods

2.1. Insects

H. peltigera moths were reared on an artificial diet as described previously [16]. 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 [16]. All females used in this study were 3.5–4.5-day-old.

2.2. Peptide synthesis

2.2.1. Chemicals for peptide synthesis

Protected amino acids, Rink amide methylbenzhydrylamine (MBHA) resin and coupling reagents were purchased from Novabiochem (Laufelfingen, Switzerland). All other reagents for peptide synthesis were purchased from Baker (Phillipsburg, NJ, USA).

2.2.2. Synthesis of PBAN, PT, LPK and MT

Hez-PBAN [53] and *Pseudaletia* (*Mythimna*) *separata* PT (Pss-PT) [32], 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 [64]. The *Leucophaea maderae* LPK (Lem-LPK) [27] and *Locusta migratoria* myotropin (Lom-MT-II) [55] were synthesized by means of the 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 [42].

2.2.3. Purification and characterization of peptides

The purity of PBAN and PT was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) on

"LichrosorbR" C-18 (LICHROCARTE 250-10 Agilent) with acetonitrile (ACN):H_2O + 0.1% trifluoroacetic acid (TFA) as described previously [5,63,64], and was found to be in the range of 90–95%. The purified peptides were characterized by time-of-flight mass spectrometry (TOF-MS) and amino acid analysis of hydrolyzates. The analytical methods and the properties of the peptides were described previously [5,63,64].

The crude peptides MT and LPK were purified on a Waters C_{18} Sep Pak cartridge and a Delta Pak C_{18} reverse-phase column (8 mm × 100 mm, 15 μm particle size, 100 Å pore size) on a Waters 510 HPLC controlled by a Millennium 2010 chromatography manager system (Waters, Milford, MA). Detection was at 214 nm at ambient temperature. Solvent A = 0.1% aqueous TFA; Solvent B = 80% aqueous ACN containing 0.1% TFA. Conditions: initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. The peptides were further purified on a Waters Protein Pak I125 column (7.8 mm × 300 mm) (Milligen Corp., Milford, MA). Conditions: flow rate: 2.0 ml/min; isocratic with solvent = 80% ACN made with 0.01% TFA. The identities of the peptide analogs were confirmed by means of MALDI-TOF-MS on a Kratos Kompact Probe MALDI-TOF MS (Kratos Analytical, Ltd., Manchester, UK). Amino acid analysis was carried out as described previously [42] and was used to quantify the peptide and to confirm its identity. The purity of MT and LPK was found to be >95%.

2.3. Pheromonotropic bioassay

2.3.1. Topical application

The cuticular scales on the ventral surface of the abdomen of 3.5–4.5-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 and 1 μl of the peptide solution was applied on 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 μl of solvent (DDW or DMSO without peptides) was applied, served as controls, to determine the basal pheromone biosynthesis at photophase induced by the vehicle itself. All experiments were carried out in parallel to experiments in which the elicitors were tested. All experiments (including controls) were carried out in 2–4 different moth colonies, to avoid the possibility that peptide penetrability is characteristic of only one colony. All experiments were performed with a minimum of seven females per treatment.

2.3.2. Peptide injection

The pheromonotropic bioassay was performed with *H. peltigera* as described previously [7]. Females injected with 100 mM phosphate buffer, pH 7.4 served as controls, to determine the basal pheromone biosynthesis at photophase. All experiments were performed with 8–10 females per treatment.

2.3.3. Gland excision

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 [7]. The pheromone content in control females did not exceed 10 ng/gland in both topical application and injection experiments. All peptides, whether injected or topically applied, were tested for their stimulatory activity, i.e., their ability to induce sex pheromone biosynthesis.

2.4. Statistical analysis

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

3. Results

The ability of four topically applied PK/PBAN peptides (PBAN, PK, LPK and MT) to stimulate sex pheromone biosynthesis is depicted in Fig. 1. The data clearly indicate that all peptides were highly potent when applied in DDW for 3 h, and were capable of stimulating sex pheromone biosynthesis in *H. peltigera* female moths to levels of 267, 132, 132 and 126 ng, respectively. The peptides were also highly potent when applied in DMSO for 3 h,

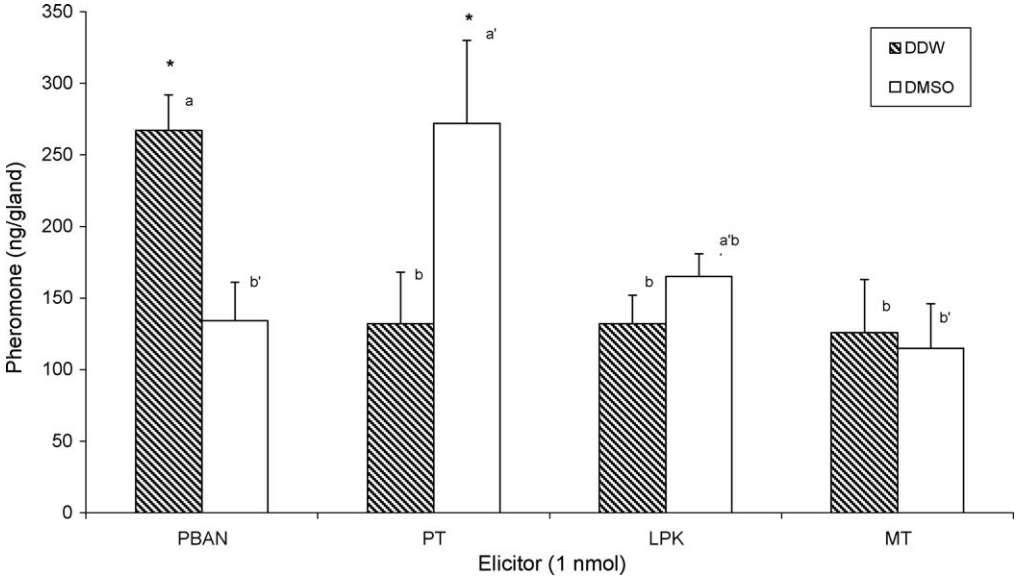

Fig. 1. Topical application of PK/PBAN peptides in DDW and DMSO. Data are presented as means ± S.E.M. of 7–18 samples. Peptides were applied at a dose of 1 nmol and glands were excised after 3 h. Statistical analysis compared between the activities (i.e. ability to stimulate sex pheromone biosynthesis) of the various peptides in each solvent separately (indicated by letters) and between the activities of a given peptide in DDW vs. DMSO (indicated by an asterisk (*)). Bars with the same letter do not differ significantly at $P < 0.05$. An asterisk (*) indicates significant differences at $P < 0.05$ between the activities of a given peptide applied in DDW or DMSO.

Table 2
Sex pheromone levels produced by application of solvents alone (with no elicitor) for different times.

Treatment	Pheromone content (ng/female)	*N*
DDW 0.5 h	3	16
DDW 1 h	0	26
DDW 2 h	1	28
DDW 3 h	1	24
DDW 6 h	0	38
DDW 8 h	0	18
DDW 10 h	0	16
DMSO 1 h	0	8
DMSO 3 h	1	8
DMSO 6 h	0	10

DDW and DMSO were applied at a volume of 1 μl and the moths were treated as described in Section 2. All experiments were carried out in parallel to experiments in which the elicitors were tested. Experiments were carried out in 2–4 different moth colonies, to avoid the possibility that peptide penetrability is characteristic of only one colony. *N* represents the number of insects that were tested in each treatment.

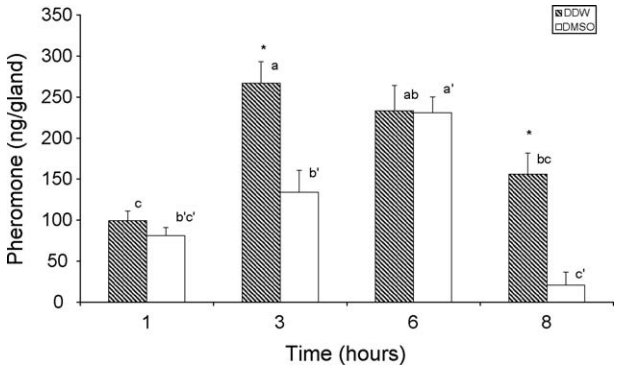

Fig. 3. Comparison of the time dependence of the activity of topically applied PBAN in DDW and in DMSO. PBAN was applied at a dose of 1 nmol. Glands were excised at the indicated times. Values represent means ± S.E.M. of 8–18 samples. Statistical analysis compared the activities of PBAN in each solvent separately (indicated by letters) at different time points. Bars with the same letter do not differ significantly at $P < 0.05$. Statistical analysis also compared PBAN activities in DDW with those in DMSO. An asterisk (*) indicates significant differences at $P < 0.05$.

when levels of 134, 272, 165 and 115 ng, respectively, were obtained. The peptides differed in their potency (i.e., ability to stimulate sex pheromone biosynthesis) and exhibited significant differences when applied in DDW or DMSO. PBAN exhibited the highest activity of all the tested peptides, in DDW, whereas PT was the most active peptide in DMSO. Comparison of the activities of each peptide in DDW and DMSO revealed that the activity of PBAN was significantly higher in DDW than in DMSO, whereas that of PT was significantly higher in DMSO; LPK and MT showed similar activities in both solvents. The pheromone content obtained in moths that were treated just with the vehicle (DDW or DMSO) for 3 h was 1 ng (Table 2).

Time response analysis indicated that PBAN was potent for up to 8 h when applied in DDW at a dose of 1 nmol (Fig. 2). The onset of the activity was quite quick stimulating pheromone content up to 85 ng pheromone 1 h post-application. The activity was highest at 3 h post-application when it generated 267 ng pheromone, and that obtained at 6 h post-application was slightly lower (233 ng) although not significantly different from that obtained at 3 h. Even at 8 h post-application the peptide stimulated sex pheromone biosynthesis to a level of 156 ng. The pheromone content obtained in moths that were treated just with the vehicle (DDW) for 0.5, 1, 2, 3, 6 and 8 h was negligible and ranged from 0 to 3 ng (Table 2).

Comparison between activities of PBAN, at 1 nmol, in DDW and in DMSO at a range of times post-application revealed that the time dependence of the peptide activity differed in the two solvents, with a faster onset and longer persistence in DDW (Fig. 3). PBAN applied in DDW reached a peak activity at 3 h whereas in DMSO it maximized at 6 h post-application. PBAN applied in DDW was still very active 3 h later (i.e., at 6 h post-application) and its activity at this time point did not differ significantly from that at 3 h. The activity of PBAN in DMSO decreased faster than that in DDW, and a marked drop in the level of pheromone content was observed between 6 and 8 h post-application (231 and 21 ng at 6 and 8 h, respectively). The activity of the peptide at 3 and 8 h post-application in DMSO was significantly lower than that following application in DDW.

Application of 1 nmol LPK in DDW or DMSO for several different durations revealed smaller differences between the time dependence of the activities than in the case of PBAN (Fig. 4). The onset of activity in both solvents was quicker than that of PBAN and reached maximal activity by 1 h post-application (97 and 108 ng, in DDW and DMSO, respectively). The overall duration of the pheromonotropic activity of LPK was similar to that of PBAN when the peptide was applied in DMSO, when it persisted for up to 6 h, but was significantly shorter when applied in DDW, as indicated by the smaller amount of pheromone found in the glands at 8 h post-application (19 and 156 ng for LPK and PBAN, respectively). Lower

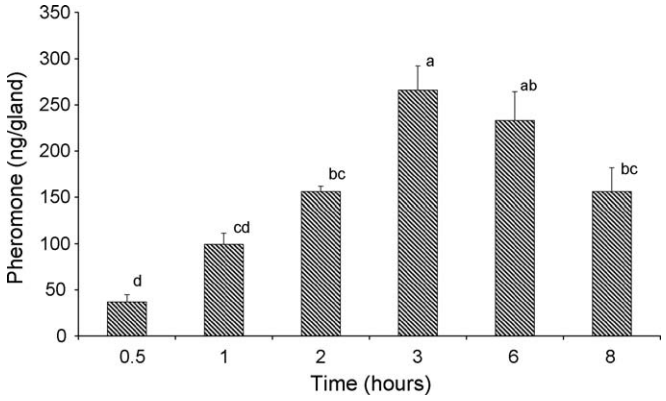

Fig. 2. Time response of topically applied PBAN in DDW. PBAN was applied at a dose of 1 nmol. Values represent means ± S.E.M. of 9–17 samples. Statistical analysis compared the activities of the peptide at all tested time points. Bars with the same letter do not differ significantly at $P < 0.05$.

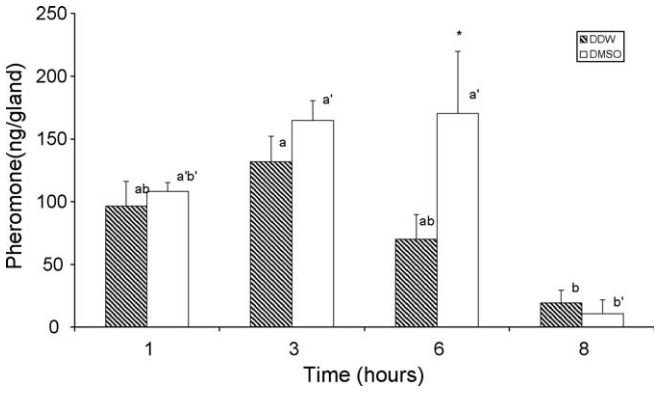

Fig. 4. Comparison of the time dependence of the activity of topically applied LPK in DDW and in DMSO. LPK was applied at a dose of 1 nmol. Glands were excised at the indicated times. Values represent means ± S.E.M. of 8–20 samples. Statistical analysis compared the activities of LPK in each solvent separately (indicated by letters) at different time points. Bars with the same letter do not differ significantly at $P < 0.05$. Statistical analysis also compared LPK activities in DDW with those in DMSO. An asterisk (*) indicates significant differences at $P < 0.05$.

Table 3
Comparison of the time dependence of the activities of topically applied and injected PBAN.

Time (h)	Topical application	Injection	
	1 nmol	1 nmol	1 pmol
0.5	37 ± 8 ($n = 9$)^{d*}	61 ± 6 ($n = 8$)^{b}	56 ± 8 ($n = 9$)^{c}
1	85 ± 12 ($n = 10$)^{cd}	77 ± 6 ($n = 9$)^{ab}	102 ± 6 ($n = 10$)^{bc}
2	156 ± 6 ($n = 10$)^{bc*}	91 ± 10 ($n = 9$)^{ab}	166 ± 22 ($n = 9$)^{ab}
3	267 ± 35 ($n = 17$)^{a*}	82 ± 12 ($n = 8$)^{ab}	181 ± 18 ($n = 8$)^{a}
6	233 ± 31 ($n = 10$)$^{ab*\#}$	114 ± 13 ($n = 9$)^{a}	73 ± 19 ($n = 10$)^{c}
8	156 ± 26 ($n = 9$)$^{bc*\#}$	61 ± 16 ($n = 9$)^{b}	38 ± 17 ($n = 10$)^{c}

PBAN in an aqueous solvent was topically applied or injected at the indicated doses. Glands were excised at the indicated times. Values represent means ± S.E.M. The vehicle itself (DDW), applied for the same time duration did not stimulate pheromone biosynthesis (Table 2). Statistical analysis compared the activities of topically applied and injected PBAN (separately) at various time points (indicated by letters). Values with the same letter do not differ significantly at $P < 0.05$. Statistical analysis also compared PBAN activities obtained by topical application or injection at each time point. A single asterisk (*) indicates significant differences (at $P < 0.05$) between topically applied and injected PBAN at 1 nmol. Significant differences between topically applied and injected PBAN at 1 pmol are indicated by symbol (#).

doses (i.e., 1–10 pmol) of topically applied LPK exhibited negligible activity. LPK (at 100 pmol) elicited formation of 93, 62 and 33 ng at 1, 2 and 3 h post-application. Similar results were obtained with PBAN and PT, which exhibited lower activities at 100 pmol (73 and 11 ng, respectively).

The ability of topically applied PBAN (at a dose of 1 nmol) to stimulate sex pheromone biosynthesis was compared with that of the injected peptide at various time points and at two doses (1 pmol and 1 nmol). As indicated in Table 3, the activity of the topically applied peptide did not differ significantly or was even significantly higher than that of the injected peptide at all time points with the exception of 30 min post-application where the injected peptide (at 1 nmol) exhibited a higher bioactivity.

The ability of topically applied LPK (at a dose of 1 nmol) to stimulate sex pheromone biosynthesis was also compared with that of the injected peptide (at a dose of 100 pmol) at various time points. As indicated in Fig. 5, the activity of the peptide was similar with both routes of application, and the pheromone content detected in the gland at 0.5, 1, 2 and 3 h after injection of the peptide (26, 60, 111, 137 ng) did not differ significantly at any time point from that obtained by topical application (28, 97, 102, 132 ng), respectively. The time course of activity differed slightly between the two administration routes: topically applied LPK reached full activity after 1 h; the injected peptide was somewhat slower, reaching maximal activity at 2 h post-injection.

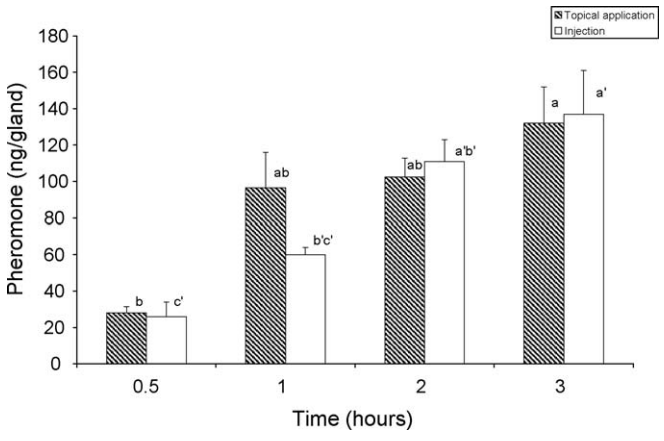

Fig. 5. Comparison of the time dependence of the activities of topically applied and injected LPK. LPK in an aqueous solvent was topically applied (at a dose of 1 nmol) or injected (at a dose of 100 pmol). Glands were excised at the indicated times. Values represent means ± S.E.M. of 7–16 samples. Statistical analysis compared the activities of topically applied or injected LPK (separately) at various time points (indicated by letters). Bars with the same letter do not differ significantly at $P < 0.05$. Statistical analysis also compared LPK activities obtained by topical application or injection at each time point. No significant differences were obtained at $P < 0.05$.

4. Discussion

The bioavailability of linear, unmodified peptides, i.e., their ability to penetrate the insect cuticle, to be delivered to their target organ and to activate the receptors was tested by topical application of four peptides of the PK/PBAN family: Hez-PBAN, Pss-PT, LPK and Lom-MT-II (Table 1). The peptides belong to the PK/PBAN family, which is a ubiquitous multifunctional family that plays a major physiological role in regulating a wide range of developmental processes in insects: pupariation [47]; diapause [29,39,58,62,65,66]; cuticular melanization [9,33]; feeding, i.e., gut muscle contraction [37,56]; and mating behavior, i.e., sex pheromone production [7,54]. Hez-PBAN is a 33-amino acid peptide [53], Pss-PT is an 18 amino acid peptide [32], LPK and Lom-MT-II are short, 8 amino acid peptides [27,55]. All of those peptides are C-terminally amidated Nps, and they share a consensus sequence (FXPRLamide, X = S, T, G or V), that constitutes their active site (Table 1 [4]). LPK is also blocked at its N-terminus by a p-Glu amino acid; the other three peptides have a free N-terminus. The bioavailability of the linear peptides was tested by evaluating their ability to stimulate sex pheromone biosynthesis following their topical application on adult *H. peltigera* female abdomens.

The present study compared the activity of the peptides in DDW and in DMSO, assessed the time dependence in the respective solvents, and compared the activity of topically applied with that of injected peptides. The choice of DMSO was based on the ability of this solvent to dissolve compounds with a wide range of polarities, and on our previous successful experience of penetration of pheromone biosynthesis precursors applied in this carrier solvent into insects [17].

The findings clearly indicated that all four linear peptides, topically applied at a dose of 1 nmol, were highly potent when applied either in an aqueous solution or in DMSO, although their activities (i.e., ability to stimulate sex pheromone biosynthesis) in the two solvents differed with PBAN being most active in DDW and PT in DMSO (Fig. 1). Hez-PBAN, at 100 pmol, was also active in both solvents, although to a lower extent. The vehicles (DDW or DMSO) themselves, applied for 3 h, did not have any effect on pheromone biosynthesis (Table 2). The activities of PBAN and PT were found to be very consistent with their hydrophilic properties: PBAN, which is a highly hydrophilic peptide, with a hydropathy index of −42.9, exhibited higher activity in DDW than in DMSO, whereas PT, which is much less hydrophilic, with a hydropathy index of −12.6, exhibited a higher activity in DMSO. At present, it is not clear how the peptides transmigrate through the cuticle. Two main routes are possible: via the intersegmental membrane and through the tracheal openings. It is possible that different elicitors chose different routes when applied in solvents of differing polarity, such as DDW and DMSO.

The activity of PBAN in DDW was relatively high (85 ng) at 1 h post-application and lasted up to 8 h (Fig. 2). The DDW itself, applied for the indicated times, did not have any effect on pheromone biosynthesis (Table 2). The time dependence pattern following application in DDW revealed a faster onset and more persistent activity than that following application in DMSO (Fig. 3). Differences were also noticed in the time to maximal activity (3 h in DDW and 6 h in DMSO), hinting that accessibility to the target of the DMSO might be slower than that of the peptide in DDW. This can be attributed to the behavior of the cuticle, which is highly hydrophobic, and serves as a reservoir from which slow release of the DMSO dissolved peptide can occur. Behavior of the cuticle as a

slow-release device has been previously demonstrated by Teal et al. [59]. The faster decline in the activity of PBAN in DMSO than in DDW may have resulted from faster clearance of the peptide from the hemolymph in the presence of the former solvent, or from a change in its conformation that would make it more accessible to proteolytic enzymes. The persistence of the activity of PBAN in DDW indicated that the peptide was very stable when applied in this solvent, despite the common notion that Nps have a short half life when administered into insects because of proteolysis [20,21,35,36,42,45]. The persistence of PBAN activity may be the result of an intrinsic or acquired biostable conformation. Such a conformation could be contributed by the beta turn in the signature sequence (FSPRLa) of the PK/PBAN family [40,41], from its binding to carrier/scavenger proteins in the hemolymph, or by formation of aggregates that acquire peptides protection against proteolytic enzymes. Alternatively, the persistence could result from low proteolytic activity in the adult female hemolymph. Indeed, a study by Weirich et al. [61] revealed that exposure of PBAN to hemolymph of *Manduca sexta* or *Helicoverpa zea* adult female moths for up to 4 h at 30 °C resulted in only a minor and very slow degradation (2–10 fmol/min/μl of hemolymph). Similar results were obtained in our laboratory where synthetic PBAN (dissolved in 0.1 M phosphate buffer, pH 7.2) was exposed to *H. peltigera* larval hemolymph, which is considered to contain much higher levels of proteolytic enzymes (unpublished data). Even if the peptide was degraded by proteolytic enzymes it might still exhibit activity if the degradation occurred in the N-terminal part of the molecule. Previous studies of the structure activity relationship (SAR) of PBAN revealed that most of the N-terminal part was not essential for bioactivity, and that the C-terminally derived hexapeptide was capable of stimulating sex pheromone production to a similar extent to that elicited by PBAN ([7,10] and references therein).

In the case of LPK, the time dependence of its activity did not differ greatly between the two tested solvents (Fig. 4), with the exception of 6 h where the DMSO applied peptide was significantly more active, and the onset of its activity in both DDW and DMSO was quicker than that of PBAN: it maximized at 1 h post-application, compared with 3 and 6 h for PBAN in DDW and DMSO, respectively. The overall duration of activity of LPK in both solvents was 6 h, which is quite remarkable for an 8 amino acid peptide. Short peptides are known to be highly susceptible to proteolytic degradation, and the relatively long lasting bioactivity could have been because of the N- and C-terminal protecting groups of the peptide (pGlu and a C-terminal amide) or because the peptide had a biostable conformation, either as discussed above, or acquired in the hemolymph. Further studies of the bioactivity of these peptides in other solvents are needed, in order to clarify the relationship between the nature of the vehicle and the bioactivity.

Comparison between the bioactivities of topically applied and injected LPK revealed that the peptide was equally potent in the two routes of application (Fig. 5). Interestingly, topically applied LPK reached full activity after 1 h and the injected peptide was somewhat slower, reaching maximal activity at 2 h post-injection. This was quite unexpected in view of the fact the injected peptide is introduced in close proximity to the target organ, whereas the topically applied peptide has to penetrate the cuticle, to be transported to the pheromone gland and to exert its activity. One possible explanation is that injection causes stress to the insect, which results in a delay in the onset of sex pheromone biosynthesis. Another possible explanation is that the injected peptides were degraded by soluble or membrane-bound peptidases released from the damaged tissue, so that the activity of the peptide was exhibited only after the enzymes had been cleared from the target area. Proof of the presence of such proteolytic enzymes associated with injection was found in one of our previous studies, in which PBAN and a hexapeptide fragment derived from its C-terminus (PBAN28-33NH_2) were tested for bioactivity in the presence and absence of a cocktail of protease inhibitors. The findings of that study demonstrated that the activity of both peptides was enhanced in the presence of the inhibitors, and that the activity of the hexapeptide persisted much longer than in their absence, and even after 1 h did not differ from that of the full-length PBAN [8]. Comparison between topically applied and injected 1 nmol dose of PBAN at various times post-application revealed much higher (i.e., 1.1–3.3-fold) pheromone content at almost all time points in the moths that received topically applied PBAN (Table 3). Evaluation of bioactivity at a lower dose (1 pmol) of injected PBAN (used in order to eliminate the possibility that the comparison is carried out at high dose of injected PBAN, which might have caused desensitization of the receptor), revealed a similar trend where the topically applied peptide exhibited an equivalent or even significantly higher bioactivity than injected PBAN.

Lower doses (i.e., 1–10 pmol) of topically applied LPK exhibited negligible activity. LPK (at 100 pmol) elicited formation of 93, 62 and 33 ng at 1, 2 and 3 h post-application. Similar results were obtained with PBAN and PT, which exhibited lower activities at 100 pmol (73 and 11 ng, respectively). It is possible that, following their introduction into the insect, the topically applied peptides became more diluted in the hemolymph than the injected ones, since the latter were applied in close proximity to the target organ. It should also be noted that the tested peptides were topically applied, so far, in only two solvents, DDW and DMSO, and use of other solvents at a wider range of time points may improve bioavailability at lower doses.

The bioavailability of PBAN and of a variety of PK/PBAN-derived analogs has been previously studied by several groups. In *H. virescens*, Nachman et al. [44] demonstrated potent pheromonotropic activity (at 60 pmol), of a topically applied carboranyl pseudotetrapeptide analog of LPK which contained a hydrophobic cage-like *o*-carborane moiety instead of Phe in the consensus C-terminal sequence; and Abernathy et al. [1] demonstrated in the same insect that an amphiphilic N-terminally modified Lom-MT-II (6-phenylhexanoic acid-Ala) was highly active (at 10 and 100 pmol) when applied in either DMSO or DDW, respectively. Topically applied LPK analogs containing C8 and C10 fatty acid chains at the N-terminal part also exhibited bioactivity in *H. virescens* at doses of 100–250 pmol and attachment of cholic acid, 1-pyrenebutyric acid or 9-fluoreneacetic acid to the N-terminus of the LPK core pentapeptide, or replacement of the Phe with hydrocinnamic acid or of the Pro with hydroxyproline (in addition to the attachment of an hexanoic acid group at the N-terminus) led to highly efficacious topically active analogs [45,46,60]. Some of these compounds were also tested for their rate of penetration through dissected pieces of cuticle, and were found to have high penetrability [59].

In all of the above studies, however, only peptides modified in a manner that attached a hydrophobic moiety to them, thus giving them an amphiphilic character, were active when applied in an aqueous solvent. Such a character conferred on them the ability to penetrate the hydrophobic insect cuticle and simultaneously to maintain the water solubility necessary to re-emerge in the insect circulatory system and to reach the receptor on the target organ. Topically applied unmodified Hez-PBAN or Lom-MT-II at doses ranging from 100 to 1000 pmol, in aqueous solution failed to elicit any bioactivity up to 3 h post-application. It is possible that the use of different moths (*H. peltigera* vs. *H. virescens*) or differences in the methods of cuticle-preparation or application of the test peptides account for the differences between the bioactivity obtained in the above studies and that obtained in our present study.

In summary, the present results include important new information on the bioavailability of unmodified linear peptides in moths, which clearly indicates that linear, polar, large and small peptides can penetrate the cuticle at a very high efficiency following contact application in aqueous solution as well as in organic solvents, and can reach their target organ without losing activity. The findings also reveal that cuticular transmigration and the time kinetics of peptides constitute a complex issue and depend on combinations of a variety of properties and factors, such as their hydrophobicity, amphiphilic properties, size, conformation, and the solvent in which they are applied. Further studies on the peptide properties that lead to high cuticular penetration are currently in progress, and their findings should be taken into consideration 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 neuroendocrinology studies and to the strategies that should be considered in development of environmentally friendly and biodegradable, rationally designed control agents, based on Np agonists or antagonists. Use of the non-invasive application route in such studies eliminates the stress and injury caused to the insect by injection which could significantly affect the *in vivo* measurement of any tested physiological parameter.

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