

Bioavailability of backbone cyclic PK/PBAN neuropeptide antagonists – inhibition of sex pheromone biosynthesis elicited by the natural mechanism in *Heliothis peltigera* females

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The bioavailability (i.e. ability to penetrate the insect cuticle, to reach the target organ and to exert bioactivity) of two backbone cyclic (BBC) pyrokinin/pheromone biosynthesis-activating neuropeptide (PK/PBAN) antagonistic peptides was tested by applying them topically to *Heliothis peltigera* females and monitoring the resulting inhibition of sex pheromone production elicited by the natural (endogenous) mechanism during scotophase. Peptides were applied at various time points before the onset of scotophase, in aqueous or organic solvents, and pheromone content was examined at the 5th or 6th hour of scotophase. Both peptides penetrated the cuticle very efficiently and inhibited sex pheromone biosynthesis elicited by the natural mechanism for up to 8 or 9 h after application. The degree of inhibition differed between solvents: those applied in double-distilled water (DDW) were more active than those applied in dimethylsulfoxide (inhibition by 53–73% and 15–38%, respectively, for BBC-25, and 46–67% and 36–40%, respectively for BBC-28). Peptides applied in dimethylsulfoxide and hexane exhibited slightly more persistent inhibitory activity than those applied in DDW. The solvents themselves did not affect sex pheromone production. Multiple applications (at –2, 0, +2 and +4 h) resulted in almost complete (87%) inhibition of sex pheromone biosynthesis, compared with 52% inhibition following a single application. The present study is the first demonstration of the ability of topically applied PK/PBAN antagonists to inhibit sex pheromone biosynthesis elicited by the natural mechanism in female moths, and provides important information on the bioavailability of BBC peptides and the mechanism responsible for sex pheromone production in these insects.

Introduction

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) peptides are members of a multifunctional family that plays a significant role in the physiology of insects. The first member of the family, leucopyrokinin, was discovered in the cockroach *Leu-*

cophaea maderae in 1986 [1], and over 30 members have been identified since then. They include pyrokinins, myotropins, PBAN, melanization and reddish coloration hormone, diapause hormone, pheromone-tropin, and peptides derived from the PBAN gene or

Abbreviations

BBC, backbone cyclic; DDW, double-distilled water; PK/PBAN, pyrokinin/pheromone biosynthesis-activating neuropeptide.

cDNA of various moths (termed pheromonotropic β and γ peptides), all of which share the common C-terminal pentapeptide, FXPRL amide (where X = S, T, G or V) (for detailed review of PK/PBAN peptides and their functions, see [2]). Functions of the PK/PBAN family include stimulation of sex pheromone biosynthesis in moths [3,4], mediation of key functions associated with feeding (gut muscle contractions) [5,6], development (embryonic diapause, pupal diapause and pupariation) [7–11] and defense (melanin biosynthesis) [12,13] in a variety of insects (moths, cockroaches, locusts and flies). Studies have shown that all of the above functions can be stimulated by more than one peptide, and that the peptides do not exhibit species specificity.

The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides elicit their effects, such as: ‘Do all peptides mediate a given function in a given insect species via one receptor or via several different receptors or receptor sub-types?’ ‘Are different functions in a given insect species mediated via the same receptor or via different receptor subtypes?’ ‘Is a given function in different insect species mediated via the same or different receptor(s)?’. As the activity of the PK/PBAN peptides has been evaluated mainly through use of synthetic peptides injected *in vivo* – an approach that may not fully reflect their endogenous (native) role – our understanding of the modes of action of this family at the cellular level, of the nature of the native (endogenous) peptides that mediate each function, and of the receptors that mediate these functions in moths and other insects is very limited and requires further investigation. Antagonists, particularly selective ones, can shed light on this issue.

However, antagonists are not only attractive for understanding the neuroendocrine control of these processes, they can also serve as novel insecticides/insect control agents. Since the PK/PBAN neuropeptides play a major role in many physiological and behavioral processes during development, reproduction, feeding and defense, their antagonists may be good candidates for insecticides/insect control agents, as they may compete with the natural elicitors and disrupt or interfere with their normal function. The high potential of such antagonists has stimulated active interest in the mode of action of this family of neuropeptides in general, and in approaches to obtain PK/PBAN antagonists in particular (for review, see [2]).

Over the past few years, we have devised several novel integrated approaches to the rational design of highly potent and metabolically stable antagonists. One such approach (termed insect neuropeptide

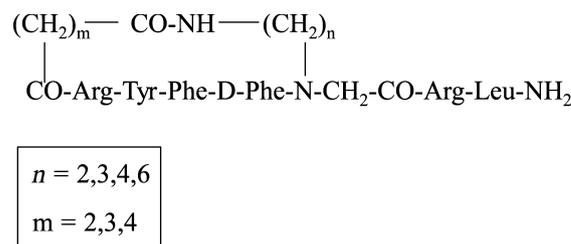


Fig. 1. General structure of the BBC d-Phe peptides.

antagonist insecticide, INAI [2]), based on substitution of L-amino acids with D-Phe followed by backbone cyclization, has led to the discovery of several highly potent linear and conformationally constrained, selective, metabolically stable backbone cyclic (BBC) pure antagonists (BBC-20, 22, 25 and 28, for which $n + m = 2 + 3$, $3 + 2$, $4 + 2$ and $6 + 2$, respectively; Fig. 1) synthesized on the basis of a sequence of a linear lead antagonist (RYF_dFPRLa) [2,14–16]. The above BBC compounds were the first identified antagonists of the PK/PBAN family and later served as a basis for the design of other antagonistic peptides based on linear amphiphilic, peptidase-resistant pseudopeptides [17].

The conformational constraint, selectivity [14] and high metabolic stability [18] of the BBC peptides enabled us to evaluate their structure–activity relationship and to analyze their ability to inhibit other functions – cuticular melanization, pupariation and hindgut contraction – mediated by PBAN and other members of the PK/PBAN family such as pheromonotropin, *Locusta migratoria* myotropin-II (Lom-MT-II) and *Leucophaea maderae* pyrokinin (Lem-PK). Particular emphasis was placed on whether elicitation of a given function by several different peptides and elicitation of several different functions by a given peptide is inhibited by the same or by different BBC peptides. All of the above experiments were carried out by co-injection of the BBC with a given elicitor [2].

Although these experiments yielded a lot of information on the structure–activity relationship of the peptides, distinguished between active (antagonistic) and inactive compounds, and even hinted at the possibility that different functions are mediated by different receptor subtypes [14], the fact that the experiments used injection-based bioassays under conditions in which the activity was elicited ‘artificially’ by a synthetic peptide raised the possibility that the results may not fully reflect the mechanisms by which these peptides act naturally.

Evaluation of the ability of some BBC peptides (BBC-20, BBC-25 and BBC-28) to inhibit sex pheromone biosynthesis elicited by the endogenous

mechanism, i.e. by the natural peptides, was also performed. In these experiments, the BBC peptides were injected 1 h before the onset of scotophase, and pheromone content in the gland was monitored hourly from the 5th to the 10th hour of scotophase. The results confirmed that these BBC peptides are potent inhibitors of the endogenous mechanism and that the inhibition of BBC-28 (Fig. 1) could last up to 11 h post-injection [18], but such experiments were not always successful, and, in many instances, control moths, injected with just the vehicle (10 mM sodium phosphate buffer, pH 7.4) without the tested peptide, produced significantly lower amounts of endogenous pheromone than found in untreated control moths – most likely because of the stress imposed on the females in the course of or as a result of the injection.

Recently, we demonstrated, for the first time, that native, unmodified linear peptides of various lengths belonging to the PK/PBAN family (PBAN, leucopyrokinin, pheromonotropin and myotropin), as well as small, linear synthetic β -substituted peptides (Ac-YFT [β^3 P]RLa, Ac-Y [β^2 homoF]TPRLa, Ac-Y [β^3 F]TPRLa and Ac- [β^3 F]FT [β^3 P]RLa) and two analogs (YFTPRLa and the acylated peptide Ac-YFTPRLa) were highly bioavailable, i.e. they could penetrate the cuticle very efficiently and reach and activate their target organ following topical application to the female moth abdomen, at a dose of 1 nmol in either double-distilled water (DDW) or dimethylsulfoxide [19,20].

In the present study, we extended our evaluation of peptide bioavailability from linear to BBC peptides, which are more stable and more hydrophobic than the linear ones, by testing the ability of topically applied BBC peptides to inhibit sex pheromone biosynthesis elicited by the endogenous (natural) mechanism. Two BBC PK/PBAN antagonistic peptides (BBC-25 and BBC-28) were tested for bioavailability by monitoring their ability to inhibit the native sex pheromone production process at various time points following their topical application to *H. peltigera* female moths in various aqueous and organic solvents, and by comparing the effects of single and multiple applications. The results revealed that both BBC peptides could penetrate the cuticle very efficiently following contact application in both aqueous and organic solvents, and could inhibit sex pheromone biosynthesis elicited by the endogenous (natural) mechanism for up to 8 or 9 h post application. The existence of potent antagonists that can inhibit the native mechanism by topical application without the need for injection represents a major advantage in the study of the mode of action of sex pheromone biosynthesis by PK/PBAN peptides, and provides important information that can contrib-

ute to the development of insecticides/control agents based on insect-neuropeptide antagonists.

Results

Endogenous pheromone biosynthesis

Examination of endogenous (native) sex pheromone levels in *H. peltigera* female moths from 1 h before the onset of scotophase until the 7th hour of scotophase indicated that no pheromone was present in the first 3 h. Pheromone was first detected at the 4th hour, reached a maximum of 154 ng in the 6th hour, and then decreased significantly in the 7th hour (Fig. 2).

Topical application of BBC-25 in various solvents and at various time points

The bioavailability of the BBC-25 peptide, i.e. its ability to penetrate the insect cuticle and to exhibit bioactivity, was examined by topical application of a 1 nmol dose of the peptide in DDW, dimethylsulfoxide or hexane 1 h before the onset of scotophase. Pheromone content was examined 6 and 7 h post-application, i.e. at the 5th and 6th hours of scotophase. The results indicated that the peptide inhibited sex pheromone biosynthesis in *H. peltigera* female moths (Fig. 3), and that there were marked differences between the various solvents and times post-application with regard to the levels of inhibition. Topical application of BBC-25 in DDW resulted in a significant decrease in pheromone content (73 and 53% at the 5th and 6th hours of scotophase, respectively), compared to that found in untreated females at these time points. Topical application of BBC-25 in dimethylsulfoxide or hexane resulted in a lower but still significant decrease in pheromone content (38 and 24%, respectively, at the 6th hour of scotophase). At 6 h post-application (5th hour of scotophase), peptides applied in dimethylsulfoxide and hexane showed weak inhibitory activities (15 and 27%, respectively), and the amount of pheromone produced by these females did not differ significantly from that found in untreated moths (Fig. 3).

Pheromone contents, as measured at the 6th hour of scotophase in females that were topically treated with 1 μ L of the solvents alone, without the tested peptide, did not differ significantly from those of untreated females, indicating that the solvents by themselves did not interfere with pheromone production (Fig. 4).

Comparison between the activities, as measured at the 5th and 6th hours of scotophase, of 1 nmol doses

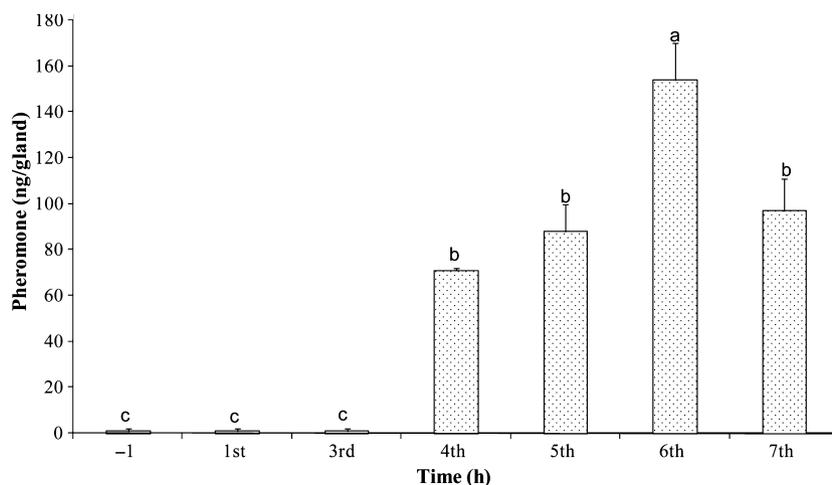


Fig. 2. Endogenous pheromone content at scotophase. Pheromone content was determined at 1 h before onset of scotophase (-1) and each hour until the 7th hour of scotophase. Values represent means \pm SEM for at least nine females. Statistical analysis was used to compare the pheromone levels at all time points. Columns with the same letter do not differ significantly at $P < 0.05$.

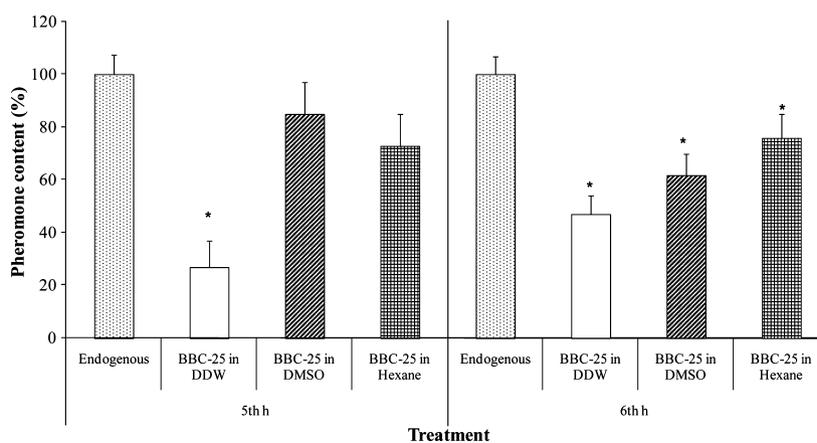


Fig. 3. Topical application of BBC-25 in several solvents. The peptide was applied at a dose of 1 nmol, and pheromone content was determined at the 5th and 6th hours of scotophase. Values represent means \pm SEM for at least 19 samples. Statistical analysis for each solvent compared pheromone content in treated females at the 5th and 6th hours of scotophase with that in untreated moths (endogenous, defined as 100%) at the same time points. Asterisks indicate a significant difference in pheromone content at $P < 0.05$. The mean pheromone contents in untreated females at the 5th and 6th hours of scotophase were 91 and 109 ng, respectively. DMSO, dimethylsulfoxide.

of BBC-25 in DDW, dimethylsulfoxide or hexane, applied 1, 2 or 3 h before the onset of scotophase, revealed inhibitory activity when the peptide was applied in DDW or dimethylsulfoxide at -1 or -2 h and in hexane at all three time points. The strongest inhibitory effects in each solvent were obtained when the peptide was applied in DDW at -1 h (53% inhibition), in dimethylsulfoxide at -2 h (53%) and in hexane at -3 h (52%) (Fig. 5).

Topical application of BBC-28 in various solvents

Evaluation of the ability of 1 nmol doses of BBC-28, applied topically in DDW and in dimethylsulfoxide, to inhibit sex pheromone biosynthesis, as measured at the 5th, 6th and 7th hours of scotophase, revealed strong inhibition when the peptide was applied in DDW, and

no activity in dimethylsulfoxide. The peptide applied in DDW inhibited pheromone synthesis by 67, 46 and 33% at the respective time points, and the pheromone levels of treated females differed significantly from those of untreated control ones (Fig. 6). In contrast to the results obtained in DDW, application of the peptide in dimethylsulfoxide resulted in small, non-significant differences in pheromone content from those in the untreated controls: 36 and 40% at the 5th and 6th hours of scotophase, respectively (Fig. 6).

Single and multiple applications of BBC-25

Comparison of the inhibitory activity of a single application of BBC-25 in dimethylsulfoxide, 2 h before the onset of scotophase, with that obtained when the peptide was applied at 2 h intervals from 2 h before the

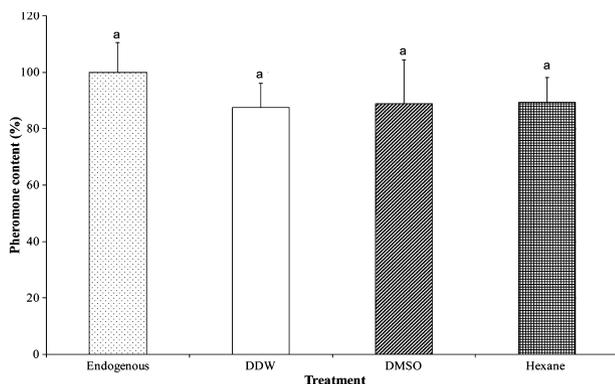


Fig. 4. Endogenous pheromone levels at the 6th hour of scotophase after topical application of 1 μ L of the various solvents without peptide. Values represent means \pm SEM for at least eight samples. Statistical analysis compared the pheromone contents of females treated with the various solvents with that of untreated females (endogenous). Bars with the same letter do not differ significantly (at $P < 0.05$). The mean pheromone content in untreated females at the 6th hour of scotophase was 65 ng.

onset of scotophase (–2) to 4 h after the onset of scotophase showed that the latter treatment achieved almost complete (87%) inhibition of pheromone biosynthesis, whereas the single application resulted in only 52% inhibition (Fig. 7). The females in the two treatments produced 15 and 64 ng of pheromone, respectively, values that are significantly different from one another, and both significantly different from the 113 ng produced by untreated females.

Discussion

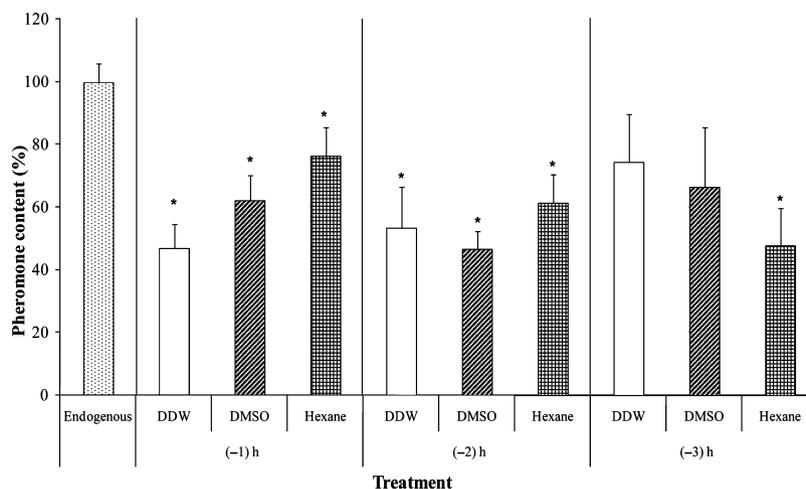
The bioavailability of two BBC PK/PBAN antagonists (BBC-25 and BBC-28) was tested by applying them topically to *H. peltigera* females and monitoring the

resulting inhibition of naturally (endogenously) elicited sex pheromone production during scotophase.

The BBC peptides used in this study are members of a backbone cyclic sub-library (the D-Phe sub-library) of conformationally constrained peptides that were synthesized on the basis of a linear lead antagonist (RYFdfPRLa) [15,21], and which had been shown to be highly potent antagonists of PK/PBAN-mediated functions in injection-based pheromonotropic, melanotropic and pupariation bioassays [14]. Backbone cyclization provides peptides with higher metabolic stability, biological activity and hydrophobicity than linear peptides [18,21,22]. Thus, BBC peptides are likely to exhibit high bioavailability. This likelihood, together with the high antagonistic activity of some BBC peptides, makes them excellent candidates for examination of their ability to penetrate the insect cuticle and subsequently inhibit sex pheromone biosynthesis.

Previous studies aimed at discovering PK/PBAN antagonists and characterizing their antagonistic properties used bioassays based on co-injection of the tested compound with a synthetic stimulator that ‘artificially’ elicited sex pheromone biosynthesis or other PK/PBAN-mediated functions (for example, see [14]). Attempts to use an injection-based bioassay to evaluate the ability of the BBC peptides to inhibit sex pheromone biosynthesis elicited by the endogenous mechanism, i.e. by the natural peptides, revealed that the injection itself affected the endogenous pheromone levels in the tested female moth, and resulted, in many cases, in production of significantly lower amounts of pheromone than those found in untreated control moths – most likely because of the stress imposed on the females during the course of the injection. The possibility that the BBC peptides might exhibit high cuticular penetrative properties suggested the use of topical

Fig. 5. Topical application of BBC-25 at various application times before the onset of scotophase (–1, –2 and –3 h), and in various solvents. The peptide was applied at a dose of 1 nmol and pheromone content was determined at the 6th hour of scotophase. Values represent means \pm SEM for at least eight samples. Statistical analysis compared pheromone content in treated females at each time point separately with that of untreated females at the 6th hour of scotophase (endogenous, defined as 100%). Asterisks indicate a significant difference at $P < 0.05$. The mean pheromone content in untreated females at the 6th hour of scotophase was 108 ng.



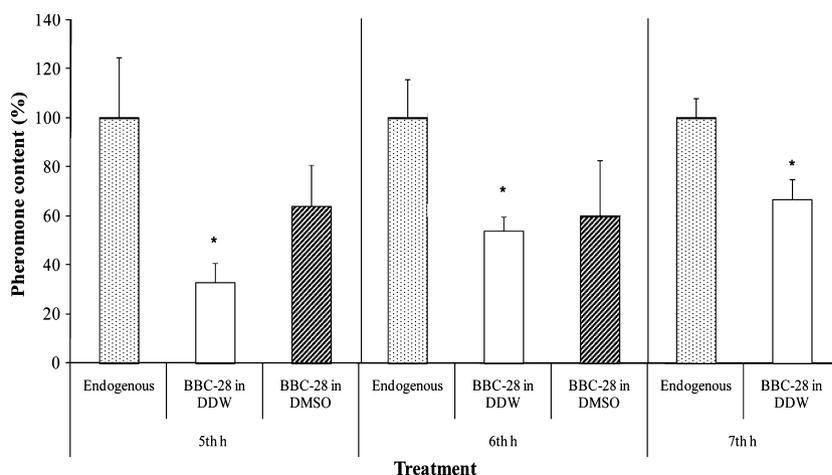


Fig. 6. Topical application of BBC-28 in various solvents. The peptide was applied at a dose of 1 nmol, and pheromone content was determined at the 5th and 6th hours of scotophase. Values represent means \pm SEM for at least 10 samples. Statistical analysis compared pheromone contents at the 5th, 6th and 7th hours of scotophase in females treated with each solvent separately with that in untreated ones (endogenous, defined as 100%) at the same time points. Asterisks indicate a significant difference in pheromone content at $P < 0.05$. The mean pheromone contents in untreated females at the 5th, 6th and 7th hours of scotophase were 93, 128 and 160 ng, respectively.

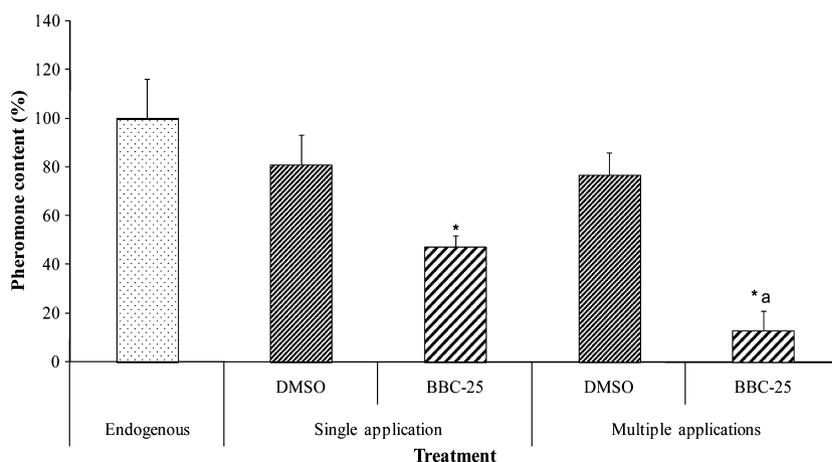


Fig. 7. Single and multiple topical application(s) of BBC-25. For the single application treatment, a 1 nmol dose of the peptide was applied 2 h before the onset of scotophase. For the multiple application treatment, 1 nmol doses of the peptide were applied 2 h before scotophase onset, at onset, and at the 2nd and 4th hours of scotophase. Pheromone content was monitored at the 6th hour of scotophase in both application modes. Values represent means \pm SEM for at least nine samples. Statistical analysis compared the pheromone content of treated females with that of untreated control ones (endogenous, defined as 100%) for each application mode separately. Asterisks indicate a significant difference at $P < 0.05$. A letter indicates a significant difference in the degree of inhibition between single and multiple applications. The mean pheromone content in untreated females at the sixth hour of scotophase was 113 ng.

application at scotophase to examine their inhibitory effect on endogenous, i.e. naturally elicited, sex pheromone production. In the present study, peptides were applied at various time points before the onset of scotophase (-1 , -2 or -3 h) in DDW, dimethylsulfoxide or hexane, and pheromone content was examined at the 5th, 6th or 7th hours of scotophase.

The results revealed that both BBC-25 and BBC-28 are able to penetrate the cuticle very efficiently following contact application in aqueous and in organic

(dimethylsulfoxide and hexane) solutions, and to inhibit endogenously elicited sex pheromone biosynthesis for up to 8 or 9 h. In most cases, BBC-28 was found to be slightly more active than BBC-25. When DDW and the other solvent vehicles were used without the tested peptides, they had no effect on the endogenous pheromone level. Interestingly, when 1 nmol doses of the peptides were tested for their inhibitory activity at photophase by co-injection with 0.5 pmol doses of PBAN as an elicitor, the inhibition levels obtained

were very similar: 77 and 55% for BBC-28 and BBC-25, respectively [16,21]. Similar inhibition levels of 70 and 54% were obtained when 1 nmol doses of BBC-28 and BBC-25, respectively, were injected into *H. peltigera* females 1 h before the onset of scotophase and inhibition of endogenous pheromone biosynthesis was evaluated at the 6th hour of scotophase [18]. The similarity between the inhibitory potency obtained by injection of the peptides in close proximity to the pheromone gland and that obtained by topical application indicates that penetration of these peptides through the cuticle is probably very high. Also interesting is the high persistence of the activity, which is consistent with the very high metabolic stability of the BBC peptides [22].

Comparison between the inhibition achieved by topical application of peptides in an aqueous solution, on the one hand, and in organic solvents (dimethylsulfoxide or hexane), on the other hand, revealed that those applied in DDW were slightly more active than those applied in dimethylsulfoxide. Interestingly, inhibition by BBC-28 in DDW was greater at the 5th hour of scotophase than that at the 6th, whereas inhibition by BBC-28 in dimethylsulfoxide was higher at the 6th hour than at the 5th. Thus, activity was more persistent when the peptide was dissolved in dimethylsulfoxide than when dissolved in DDW, although the inhibitory activity in the former solvent was lower. One possible explanation for such differences is that peptides applied in dimethylsulfoxide were more strongly retained in the hydrophobic cuticle than those applied in DDW, which led to slower release and more extended persistence. It should be noted, however, that the end-point activity measured in the above experiments, i.e. the pheromone content, represents an integrated result of the abilities of the peptide to be retained and to penetrate the cuticle, to be transported to the target organ, and to activate it. As all the relevant parameters may be affected by the administered solvent, it is difficult to determine which step is affected most by the use of a hydrophobic or an aqueous solvent.

One important parameter that has to be taken into account in experiments that focus on inhibition of the endogenous mechanism relates to our inability to determine the exact time course of the events that precede sex pheromone biosynthesis: the time point(s) and time course of the release of natural elicitor(s), the kinetics of receptor activation, and the time course of the subsequent events leading to the onset of sex pheromone biosynthesis in the gland. In contrast to injection-based assays, in which the timing of elicitation is known and the time course of the events leading to pheromone production can be determined, enabling precise determination of the optimal timing for injection

of an antagonist to obtain a maximal response, the only parameter that can be measured in 'endogenous' experiments is the pheromone content at any given time point during scotophase (see Fig. 2). Therefore, the optimum timing of application of an antagonist in such experiments has to be determined experimentally. In a previous study that used an injection-based bioassay, we found that injection of BBC peptides 1 h before the onset of scotophase resulted in inhibition of endogenous sex pheromone production by 54–70% at the 6th hour of scotophase [18]. In the present study, we used this time point as well as two earlier points, i.e. 2 and 3 h before scotophase (–2 h and –3 h, respectively) to test the inhibitory effects of topically applied BBC-25 in various solvents. The results revealed that topically applied BBC-25 inhibited sex pheromone biosynthesis for up to 8–9 h post-application, but the magnitude of this effect differed between the solvents. Peptide applied in DDW was most active when applied at –1 h, that in dimethylsulfoxide at –2 h, and that in hexane at –3 h. These differences between solvents may result from longer retention in the cuticle of the peptide dissolved in the hydrophobic solvents (dimethylsulfoxide and hexane) than of that dissolved in DDW.

As indicated above, in almost all experiments we carried out, regardless of the time of application, solvent used or route of administration (topical application or injection), the extent of inhibition by BBC-25 did not exceed 55%. Such partial inhibition could have resulted from low dosage of the peptide, or, alternatively, from the existence of more than one natural elicitor or receptor that mediate(s) sex pheromone biosynthesis, with only one of the various possible elicitor/receptor interactions inhibited by the peptide. The involvement of several receptor sub-types in pheromone biosynthesis in *H. peltigera* females has been proposed previously, in light of the ability of various BBC peptides to inhibit sex pheromone biosynthesis elicited by synthetic PBAN but not by synthetic pheromonotropin, leucopyrokinin or myotropin [2,14]. This hypothesis, however, was based on 'artificial' stimulation of sex pheromone biosynthesis. The present experimental paradigm enabled us to further examine this issue by addressing the natural endogenous mechanism, and to determine whether BBC-25 is a selective inhibitor of only one pheromonotropic mechanism. Interestingly, multiple applications of BBC-25 at several time points prior to and during scotophase (1 nmol at –2, 0, +2 and +4 h) resulted in almost complete (85%) inhibition of sex pheromone biosynthesis, compared with up to 53% inhibition obtained in a single application, indicating that the incompleteness

of inhibition did not result from selectivity of the antagonist towards one out of several possible mechanisms, but rather from the low dose used under the test conditions. However, this does not exclude the possibility that more than one elicitor/receptor may mediate sex pheromone biosynthesis, but rather indicates that the peptide might inhibit more than one such combination – if they exist. Other BBC antagonists are being evaluated with this aspect in mind.

Recently, we reported that native, β -amino acid-modified and unmodified linear agonistic peptides of various lengths (6–33 amino acids) and polarities, belonging to the PK/PBAN family, were highly bioavailable and could stimulate sex pheromone biosynthesis following topical application on female *H. peltigera* moths at a dose of 1 nmol [19,20]. In another study, we demonstrated that a linear antagonist Hex-Suc-A[dF]PRLa can penetrate the cuticle of a Heliothinae insect [17]. Currently it is difficult to compare the bioavailability of the linear peptides with that of the cyclic peptides, in terms of cuticular penetrability, because the differing natures of the peptides (agonists versus antagonists) necessitates differing experimental set-ups. Further studies of the bioavailability of linear antagonists and comparison of their activities with those of the BBC peptides are currently in progress.

In summary, the present study demonstrated the ability of topically applied PK/PBAN antagonists to penetrate the cuticle, to reach the pheromone gland, and to inhibit sex pheromone biosynthesis elicited by the natural endogenous mechanism in female moths. The present results represent a significant addition to the approaches used in moth neuroendocrine studies, in that they demonstrate that non-invasive application routes, which eliminate the stress and injury caused to the insect by injection, can be implemented and can provide more accurate *in vivo* measurements. These results also add important information regarding strategies that should be considered in development of environmentally friendly, biodegradable, rationally designed control agents based on neuropeptide agonists or antagonists, and may lead to dramatic simplification of these strategies.

Experimental procedures

Insects

Heliothis peltigera moths were reared on an artificial diet as described previously [23]. 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 sup-

plied with a 10% sugar solution. Moth populations were refreshed every year using males caught in the wild by means of pheromone traps, as described previously [23]. All females used in this study were 3–4 nights old. Experiments were performed on four *H. peltigera* colonies that had been reared for various lengths of time in the laboratory, to exclude the possibility that the results might represent a single moth colony.

Synthesis of the BBC peptides

BBC peptides were synthesized by the SMPS ‘tea bags’ methodology, on Rink Amide methylbenzhydrylamine resin by means of FastMocTM (Fmoc) chemistry, as described previously [16,21], and were purified under previously described conditions [16]. Two BBC peptides were examined: BBC-25 ($n = 6, m = 2$) and BBC-28 ($n = 4, m = 2$) (Fig. 1).

Pheromonotropic bioassay

The cuticular scales on the ventral surface of the abdomen of 3–4-night-old *H. peltigera* females at scotophase were gently removed by rubbing the abdomen with a cotton bud. The peptides were dissolved in double-distilled water (DDW), dimethylsulfoxide (BDH, London, UK) or *n*-hexane (Bio-Lab, Jerusalem, Israel), and 1 μ L of solution containing 1 nmol of the peptide 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 using 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. Untreated females were used to determine the natural (endogenous) level of pheromone biosynthesis at scotophase (control), and females that had received 1 μ L of solvent (DDW, dimethylsulfoxide or hexane) without the tested peptides (negative control) were used to determine the effect of the vehicle on pheromone content. All experiments were performed with a minimum of eight females per treatment. The peptides were tested for their inhibitory activity, i.e. their ability to suppress sex pheromone biosynthesis.

Pheromone gland excision and extraction

Pheromone glands of treated and untreated females were excised at various times post-treatment (6–9 h), and sex pheromone was extracted and quantified by capillary gas chromatography as described previously [24].

Statistical analysis

All results were subjected to one-way ANOVA. The significance of differences among means was evaluated using the

Tukey–Kramer honestly significant difference (HSD) test at $P < 0.05$. The data are presented as means \pm SEM.

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References

- Holman GM, Cook BJ & Nachman RJ (1986) Isolation, primary structure and synthesis of a blocked myotropic neuropeptide isolated from the cockroach, *Leucophaea maderae*. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **85**, 219–224.
- Altstein M & Hariton A (2009) Rational design of insect control agents: the PK/PBAN family as a study case. In *Biorational Control of Arthropod Pests: Application and Resistance Management* (Ishaaya I & Horowitz R eds), pp. 49–81. Springer, New York.
- Matsumoto S, Fonagy A, Kurihara M, Uchiumi K, Nagamine T, Chijimatsu M & Mitsui T (1992) Isolation and primary structure of a novel pheromonotropic neuropeptide structurally related to leucopyrokinin from the armyworm larvae, *Pseudaletia separata*. *Biochem Biophys Res Commun* **182**, 534–539.
- Raina AK & Klun JA (1984) Brain factor control of sex-pheromone production in the female corn-earworm moth. *Science* **225**, 531–533.
- Nachman RJ, Holman GM & Cook BJ (1986) Active fragments and analogs of the insect neuropeptide leucopyrokinin: structure–function studies. *Biochem Biophys Res Commun* **137**, 936–942.
- Schoofs L, Holman GM, Hayes TK, Nachman RJ & Deloof A (1991) Isolation, primary structure, and synthesis of locustapyrokinin – a myotropic peptide of *Locusta migratoria*. *Gen Comp Endocr* **81**, 97–104.
- Žd'árek J, Myska P, Zemek R & Nachman RJ (2002) Mode of action of an insect neuropeptide leucopyrokinin (LPK) on pupariation in fleshfly (*Sarcophaga bullata*) larvae (Diptera: Sarcophagidae). *J Insect Physiol* **48**, 951–959.
- Imai K, Konno T, Nakazawa Y, Komiya T, Isobe M, Koga K, Goto T, Yaginuma T, Sakakibara K, Hasegawa K *et al.* (1991) Isolation and structure of diapause hormone of the silkworm, *Bombyx mori*. *Proc Jpn Acad Ser B Phys Biol Sci* **67**, 98–101.
- Nachman RJ, Holman GM, Schoofs L & Yamashita O (1993) Silkmoth diapause induction activity of myotropic pyrokinin (FXPRLamide) insect neuropeptides. *Peptides* **14**, 1043–1048.
- Nachman RJ, Zdarek J, Holman GM & Hayes TK (1997) Pupariation acceleration in fleshfly (*Sarcophaga bullata*) larvae by the pyrokinin/PBAN neuropeptide family – structure–activity relationships. *Ann NY Acad Sci* **814**, 73–79.
- Xu WH & Denlinger DL (2003) Molecular characterization of prothoracicotropic hormone and diapause hormone in *Heliothis virescens* during diapause, and a new role for diapause hormone. *Insect Mol Biol* **12**, 509–516.
- Altstein M, Gazit Y, Ben Aziz O, Gabay T, Marcus R, Vogel Z & Barg J (1996) Induction of cuticular melanization in *Spodoptera littoralis* larvae by PBAN/MRCH: development of a quantitative bioassay and structure function analysis. *Arch Insect Biochem* **31**, 355–370.
- Matsumoto S, Kitamura A, Nagasawa H, Kataoka H, Orikasa C, Mitsui T & Suzuki A (1990) Functional diversity of a neurohormone produced by the subesophageal ganglion: molecular identity of melanization and reddish coloration hormone and pheromone biosynthesis activating neuropeptide. *J Insect Physiol* **36**, 427–432.
- Altstein M, Ben-Aziz O, Zeltser I, Bhargava K, Davidovitch M, Strey A, Pryor N & Nachman RJ (2007) Inhibition of PK/PBAN-mediated functions in insects: discovery of selective and non-selective inhibitors. *Peptides* **28**, 574–584.
- Zeltser I, Gilon C, Ben-Aziz O, Scheffler I & Altstein M (2000) Discovery of a linear lead antagonist to the insect pheromone biosynthesis activating neuropeptide (PBAN). *Peptides* **21**, 1457–1465.
- Zeltser I, Ben-Aziz O, Scheffler I, Bhargava K, Altstein M & Gilon C (2001) Insect neuropeptide antagonist. Part II. Synthesis and biological activity of backbone cyclic and precyclic PBAN antagonists. *J Peptide Res* **58**, 275–284.
- Nachman RJ, Teal PEA, Ben-Aziz O, Davidovitch M, Zubrzak P & Altstein M (2009) An amphiphilic, PK/PBAN analog is a selective pheromonotropic antagonist that penetrates the cuticle of a heliothine insect. *Peptides* **30**, 616–621.
- Altstein M (2003) Novel insect control agents based on neuropeptide antagonists – the PK/PBAN family as a case study. *J Mol Neurosci* **22**, 147–157.
- Hariton A, Ben-Aziz O, Davidovitch M, Nachman R & Altstein M (2009) Bioavailability of insect neuropeptides: the PK/PBAN family as a case study. *Peptides* **30**, 1034–1041.
- Hariton A, Ben-Aziz O, Davidovitch M, Zubrzak P, Nachman RJ & Altstein M (2009) Bioavailability of

- β -amino acid and C-terminally derived PK/PBAN analogs. *Peptides* **30**, 2174–2181.
- 21 Altstein M, Ben-Aziz O, Daniel S, Scheffer I, Zeltser I & Gilon C (1999) Backbone cyclic peptide antagonists, derived from the insect pheromone biosynthesis activating neuropeptide, inhibit sex pheromone biosynthesis in moths. *J Biol Chem* **274**, 17573–17579.
- 22 Altstein M (2001) Insect neuropeptide antagonists. *Biopolymers* **60**, 460–473.
- 23 Dunkelblum E & Kehat M (1989) Female sex-pheromone components of *Heliothis peltigera* (Lepidoptera: Noctuidae). Chemical identification from gland extracts and male response. *J Chem Ecol* **15**, 2233–2245.
- 24 Altstein M, Dunkelblum E, Gabay T, Ben Aziz O, Schaffer I & Gazit Y (1995) PBAN-induced sex-pheromone biosynthesis in *Heliothis peltigera*: structure, dose, and time-dependent analysis. *Arch Insect Biochem* **30**, 307–319.