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Peptides 25 (2004) 1491–1501
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Review

Role of neuropeptides in sex pheromone production in moths☆

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Received 15 March 2004; accepted 9 June 2004
Available online 21 August 2004

Abstract

Sex pheromone biosynthesis in many moth species is controlled by a cerebral neuropeptide, termed pheromone biosynthesis activating neuropeptide (PBAN). PBAN is a 33 amino acid C-terminally amidated neuropeptide that is produced by neuroendocrine cells of the subesophageal ganglion (SEG). Studies of the regulation of sex pheromone biosynthesis in moths have revealed that this function can be elicited by additional neuropeptides all of which share the common C-terminal pentapeptide FXPRL-amide (*X = S, T, G, V*). In the past two decades extensive studies were carried out on the chemical, cellular and molecular aspects of PBAN and the other peptides (termed the pyrokinin (PK)/PBAN family) aiming to understand the mode of their action on sex pheromone biosynthesis. In the present review we focus on a few of these aspects, specifically on the: (i) structure–activity relationship (SAR) of the PK/PBAN family, (ii) characterization of the PK/PBAN receptor and (iii) development of a novel strategy for the generation of PK/PBAN antagonists and their employment in studying the mode of action of the PK/PBAN peptides.

Keywords: PBAN; PBAN-receptor; Moths; Sex pheromones; Insect neuropeptides; Neuropeptide antagonists; Rational design

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☆ Contribution from the Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel, No. 504/04, 2004 series.
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doi:10.1016/j.peptides.2004.06.020

1. Introduction

1.1. Sex pheromones in Lepidoptera

The sexual communication between sexes in Lepidopteran species is mediated mainly by sex pheromones, which are volatile compounds used by the female to attract potential mates from a distance [40]. Sex pheromones play an important role in the elicitation of mating behavior in moths and are, therefore, crucial for successful mating. Understanding the mechanisms that underlie sex pheromone production is, thus, of major interest and importance.

Sex pheromones in Lepidopteran species are synthesized by females in a specialized gland, which is a modification of the inter-segmental membrane (ISM) located between the eighth and ninth abdominal segments [68,82]. The pheromone-producing cells are epithelial cells, overlaid by a modified ISM cuticle which, in most Lepidopterans, is produced by the cells themselves. The pheromone is produced within the epithelial cells, transported through the cuticle via special porous cuticular spines and disseminated from the surface [68,82].

Several hundred Lepidopteran sex pheromones, occurring in virgin female gland extracts and volatiles, have been identified in the past three decades and their modes of action have been studied extensively. Most Lepidopteran sex pheromones are C10–C18 aliphatic unsaturated aldehydes, alcohols or acetates and their structural diversity is indicated by differences in the number of carbon atoms, in the positions and configurations of the olefinic bonds and in the nature of the functional groups [15,95]. Most pheromones are blends of several components, among which major or minor constituents play important roles in the elicitation of the mating behavior. Mating in moths usually occurs during a discrete period of the photophase/scotophase cycle, and in most cases is nocturnal. Sex pheromones are crucial for successful mating and for maintaining reproductive isolation among closely related species sharing a common ecological niche.

1.2. Neuroendocrine control of sex pheromone biosynthesis in moths

Sex pheromone biosynthesis in moths is affected by a variety of exogenous and endogenous factors such as temperature, photoperiod, host plants, age and mating, as well as by endocrine and neuroendocrine factors. A major breakthrough in our understanding of the neuroendocrine mechanism involved in sex pheromone production occurred in 1984, when Raina and Klun [77] first reported that pheromone production in female *Helicoverpa* (then *Heliothis*) *zea* moths is controlled by a cerebral neuropeptide, which was termed pheromone biosynthesis activating neuropeptide (PBAN). Direct evidence for the involvement of a neuropeptide from the cerebral complex in sex pheromone biosynthesis was first demonstrated by a simple and sensitive bioassay (pheromonotropic assay) that was developed by the same group and enabled to determine its presence and activity [77]. The assay involved injection of extracts that contained a pheromonotropic factor into females that do not produce pheromone (either as a result of their ligation between the head and the thorax or their use during photophase, when sex pheromone is not produced) followed by analysis of the pheromone content in the gland at various times post-injection, by capillary gas chromatography [77].

2. PBAN and the PK/PBAN family

2.1. Isolation and identification of PBAN and other pheromonotropic peptides

The availability of the pheromonotropic bioassay enabled in 1989 to isolate and characterize PBAN from *H. zea* [79]. PBAN was found to be a 33-amino acid. C-terminally amidated neuropeptide and the peptide was termed Hez-PBAN (nomenclature according to Raina and Gäde), [78]. Since 1989 the primary sequence of PBAN has been determined in numerous other moth species (*Bombyx mori* [53], *Lymantria dispar* [60], *Helicoverpa assulta* [16], *Agrotis ipsilon* [27], *Mamestra brassicae* [47] and *Spodoptera littoralis* [45]), either by sequencing of the purified neuropeptide or from cloned cDNA or gene sequence (for review see [32,74,75], and Table 1). Since its first discovery, PBAN has been reported to control sex pheromone biosynthesis in many other moth species, and the peptide itself has been found in many Lepidopteran species as well as in other insect orders [32,74,75]. Although it is generally agreed that pheromone production in many Lepidopteran species is controlled by PBAN and possibly other members of the family (see below), not all moths utilize PBAN as a pheromonotropic hormone. A few examples of alternative mechanisms are presented in a recent review by Rafaeli and Jurenka [75].

Studies of the regulation of sex pheromone biosynthesis in moths have revealed that this function can be elicited by additional neuropeptides isolated from various insects, all of which share the common C-terminal pentapeptide FXPRL-amide (*X* = *S*, *T*, *G* or *V*) [1,30,55,56,94]. Among these peptides are the pyrokinins (PKs) (Lem-PK, Lom-PK-I and Lom-PK-II) and the myotropins Lom-MT-I to IV (myotropic peptides isolated from the cockroach *Leucophaea maderae* and the migratory locust, *Locusta migratoria*) [63,64,88,89] and a myotropic peptide from *Schistocerca gregaria* (Scg-MT-1) [97]. For details of the amino acid sequences of these peptides see Table 1. Additional peptides that were found to stimulate sex pheromone biosynthesis are the pheromonotropin (Pss-PT) an 18-amino acid peptide isolated from *Pseudaletia* (*Mythimna*) *separata* [62] and diapause hormone (DH) isolated from the silk-worm, *B. mori* [46], *H. zea* [58], *H. assulta* [16], *A. ipsilon* [27] and *S. littoralis* [45]. All of these peptides were found to contain the “signature” C-terminal sequence and have been designated the PK/PBAN family. In the past few years additional peptides were added

Table 1
Amino acid sequence of PBAN and other peptides of the PK/PBAN family.

Code name	Insect species	Amino acid sequence
Hez-PBAN	*Helicoverpa zea*	LSDDMPATPADQEMYRQDPEQIDSRTKY**F**S**PRL**-**NH**$_2$
Bom-PBAN-I/MRCH	*Bombyx mori*	LSEDMPATPADQEMYQPDPEEMESRTRY**F**S**PRL**-**NH**$_2$
Bom-PBAN-II	*Bombyx mori*	RLSEDMPATPADQEMYQPDPEEMESRTRY**F**S**PRL**-**NH**$_2$
Lyd-PBAN	*Lymantria dispar*	LADDMPATMADQEVYRPEPEQIDSRNKY**F**S**PRL**-**NH**$_2$
Has-PBAN	*Helicoverpa assulta*	LSDDMPATPADQEMYRQDPEQIDSRTKY**F**S**PRL**-**NH**$_2$
Agi-PBAN	*Agrotis ipsilon*	LADDTPATPADQEMYRPDPEQIDSRTKY**F**S**PRL**-**NH**$_2$
Mab-PBAN	*Mamestra brassicae*	LADDMPATPADQEMYRPDPEQIDSRTKY**F**S**PRL**-**NH**$_2$
Spl-PBAN	*Spodoptera littoralis*	LADDMPATPADQELYRPDPDQIDSRTKY**F**S**PRL**-**NH**$_2$
Bom-DH	*Bombyx mori*	TDMKDESDRGAHSERGALC**F**G**PRL**-**NH**$_2$
Hez-DH	*Helicoverpa zea*	NDVKDGAASGAHSDRLGLW**F**G**PRL**-**NH**$_2$
Has-DH	*Helicoverpa assulta*	NDVKDGAASGAHSDRLGLW**F**G**PRL**-**NH**$_2$
Agi-DH	*Agrotis ipsilon*	NDVKDGGADRAHSDRGGMW**F**G**PRL**-**NH**$_2$
Spl-DH	*Spodoptera littoralis*	NEIKDGGSDRGAHSDRAGLW**F**G**PRL**-**NH**$_2$
Lom-PK-I	*Locusta migratoria*	pEDSGDGWPQQP**F**V**PRL**-**NH**$_2$
Lom-PK-II	*Locusta migratoria*	pESVPT**F**T**PRL**-**NH**$_2$
Lem-PK	*Leucophaea maderae*	pETS**F**T**PRL**-**NH**$_2$
Pea-PK-4	*Periplaneta americana*	GGGGSGETSGMW**F**G**PRL**-**NH**$_2$
Pea-PK-5	*Periplaneta americana*	SESEVPGMW**F**G**PRL**-**NH**$_2$
Drm	*Drosophila melanogaster*	TGPSASSGLW**F**G**PRL**-**NH**$_2$
Lom-MT-I	*Locusta migratoria*	GAVPAAQ**F**S**PRL**-**NH**$_2$
Lom-MT-II	*Locusta migratoria*	EGD**F**T**PRL**-**NH**$_2$
Lom-MT-III	*Locusta migratoria*	RQQP**F**V**PRL**-**NH**$_2$
Lom-MT-VI	*Locusta migratoria*	RLHQNGMP**F**S**PRL**-**NH**$_2$
Scg-MT-1	*Schistocerca gregaria*	GAAPAAQ**F**S**PRL**-**NH**$_2$
Pss-PT	*Pseudaletia separata*	KLSYDDKVFENVE**F**T**PRL**-**NH**$_2$
Hez-β-NP	*Helicoverpa zea*	SLAYDDKSFENVE**F**T**PRL**-**NH**$_2$
Has-β-NP	*Helicoverpa assulta*	SLAYDDKSFENVE**F**T**PRL**-**NH**$_2$
Agi-β-NP	*Agrotis ipsilon*	SLSYEDKMFDNVE**F**T**PRL**-**NH**$_2$
Mab-β-NP	*Mamestra brassicae*	SLAYDDKVFENVE**F**T**PRL**-**NH**$_2$
Spl-β-NP	*Spodoptera littoralis*	SLAYDDKVFENVE**F**T**PRL**-**NH**$_2$
Hez-γ-NP	*Helicoverpa zea*	TMN**F**S**PRL**-**NH**$_2$
Has-γ-NP	*Helicoverpa assulta*	TMN**F**S**PRL**-**NH**$_2$
Agi-γ-NP	*Agrotis ipsilon*	TMN**F**S**PRL**-**NH**$_2$
Mab-γ-NP	*Mamestra brassicae*	TMN**F**S**PRL**-**NH**$_2$
Spl-γ-NP	*Spodoptera littoralis*	TMN**F**S**PRL**-**NH**$_2$
Bom-γ-NP	*Bombyx mori*	TMS**F**S**PRL**-**NH**$_2$

Bold letters indicate conserved amino acid sequences. DH, diapause hormone; MRCH, melanization and reddish coloration hormone; PBAN, pheromone biosynthesis activating neuropeptide; PT, pheromonotropin; PK, pyrokinin; MT, myotropin; NP, neuropeptide.

to be part of the PK/PBAN family. Among those are two PK peptides from *Periplaneta Americana* (Pea-PK-5 and Pea-PK-6) [70,71], a peptide from *Drosophila melanogaster* [17] and peptides derived from the PBAN gene or cDNA of various moths: *H. eaz* [58], *B. mori* [50], *H. assulta* [16], *A. ipsilon* [27], *M. brassicae* [47] and *S. littoralis* [45] (termed pheromonotropic β and γ peptides, with the β peptide bearing high homology with Pss-PT). Recently a few additional peptides sharing just the PRL-amide C-terminal sequence were added to the PK/PBAN family. Those are not listed in Table 1 and further information on their origin and sequence can be found in Rafaeli and Jurenka [75].

The PK/PBAN family is a multifunctional family of peptides that plays a major role in the physiology of insects. In addition to their ability to stimulate sex pheromone biosynthesis in moths, they mediate key functions associated with feeding (gut muscle contractions) [63,88], development (pupariation and diapause) [46,65,66] and defense (melanin biosynthesis) [4,61] in a variety of insects (moths, cockroaches, locusts and flies). Currently, over 30 peptides have been identified (including pyrokinins, myotropins, PBAN, melanization and reddish coloration hormone (MRCH), diapause hormone and pheromonostatin). Studies performed in several laboratories, including ours, have shown that all of the above additional functions can be stimulated by more than one peptide, and that the peptides do not exhibit species specificity (for a detailed review see Gäde [32], Rafaeli [74] and Rafaeli and Jurenka, [75]). The involvement of PK/PBAN neuropeptides in the above functions was demonstrated by means of a variety of in vivo and in vitro bioassays (e.g., the above mentioned pheromonotropic bioassay as well as melanotropic, egg diapause, pupariation and myotropic assays) that were developed and optimized in several laboratories [4,33,39,61,65,66,77,88,89,99].

The discovery of PBAN and the other PK/PBAN peptides, and the availability of the above bioassays led to a burst of studies on their isolation and identification, gene expression, biosynthesis, distribution, release and metabolism as well as on their modes of action (e.g., route of transport and target organ, cellular activity and second messenger mediation, effect on the pheromonal biosynthetic pathways, etc.). In the present review we summarize three topics which are the focus of our studies: (i) the structure–activity relationship (SAR) of the PK/PBAN family, (ii) characterization of the PK/PBAN receptor and (iii) development of a novel strategy for the generation of PK/PBAN antagonists and their employment in studying the mode of action of the PK/PBAN peptides. Additional topics are covered in two comprehensive recent reviews [74,75] and the reader is referred to them for further information.

2.2. Structure–activity relationship of the PK/PBAN family

Identification of the amino acid sequences of PBAN and of other members of the PK/PBAN family enabled to perform detailed SAR studies using synthetic peptides derived from their sequences. Studies on a variety of moth species have shown that the C-terminal region of the neuropeptide is essential for the pheromonotropic activity and that within the C-terminal region, the signature pentapeptide (FXPRL-amide; *X* = *S*, *T*, *G* or *V*) represents the minimal sequence required for induction of pheromonotropic activity, although in most cases its activity was lower than that of full-length PBAN. The amide group and the X position were shown to be of major importance [2–6,54,64,67,80,81].

The N-terminal part of the molecule was found to be much less important for the onset of pheromonotropic activity [2,53,55,80]. By using a variety of Hez-PBAN-derived fragments at a range of doses and at various times post-injection, we were able to demonstrate, on *Heliothis peltigera*, that peptides lacking 12 and even 16 amino acids from their N-terminus were as potent as the full-length PBAN, and that a C-terminal derived hexapeptide that contained the signature sequence (YFSPRL-amide) was capable of stimulating sex pheromone production to a similar extent to PBAN1-33NH_2 when its activity was analyzed at short post-injection times [3], indicating that the hexapeptide constitutes the biologically active site of the neuropeptide.

Structure–function studies were also performed on other insect neuropeptides that contain the PBAN pentapeptide C-terminal region (PKs, Bom-DH and Pss-PT). All of these peptides showed pheromonotropic activity, and confirmed the importance of the C-terminal region in its onset [1,30,56,65,89]. The ability of a variety of peptides to stimulate sex pheromone production hinted at the possible existence of multiple pheromonotropic mechanisms which may be mediated by multiple PBAN receptors or alternatively, the existence of only one mechanism mediated by the C-terminal part of the PK/PBAN neuropeptides.

2.3. The PBAN target organ and receptor

The target tissue of PBAN has been a disputed issue for many years. Several morphological, histochemical and biochemical studies clearly identified the pheromone gland as the prime target of PBAN [28,72,90]. These studies demonstrated that in vitro gland cultures or preparations of ovipositor tips from a variety of insects could easily be stimulated to produce sex pheromone by application of brain extracts or of synthetic PBAN. Anatomical evidence provided direct proof for the existence of PBAN release sites, presumably in the region of the pheromone gland [25,36,52], and recent studies demonstrated that viable pheromone gland cell clusters from the ISM could produce pheromone in response to a pheromonotropic peptide [31].

Other studies, however, indicated other organs as alternative targets. For example, Teal et al. [93] demonstrated that the prime target of PBAN is the terminal abdominal ganglion (TAG), which in turn provides a signal that elicits pheromone production by the pheromone gland. This theory was supported by the findings of Christensen et al. [19,20], who demonstrated that in the absence of PBAN, sex pheromone production in *H. zea* and *H. virescens* is elicited by the biogenic amine octopamine in an age- and photoperiod-dependent manner. These workers suggested that PBAN activates the TAG that in turn secretes octopamin, which is the factor that activates the pheromone gland.

The bursa copulatrix has also been suggested as a potential target for PBAN. In a study on *Argyrothaenia velutinana*, Jurenka et al. [49] demonstrated that abdomen cultures responded to a much greater extent than the pheromone gland to exogenously applied synthetic PBAN, and that the bursa copulatrix was essential for a full stimulatory response to PBAN. The study suggested that a pheromonotropic factor, other than PBAN, originating in the bursa copulatrix, is essential for pheromonotropic activity and that the role of PBAN is to stimulate the release of such a factor. The involvement of a bursal factor in the pheromonotropic activity has been reported in other moths as well [26]. Furthermore, in vitro experiments on pheromone glands of *Trichoplusia ni*, *Agrotis segatum*, *A. velutinana* and the pink bollworm *Pectinophora gossypiella*, failed to show pheromone biosynthesis in response to the application of brain-SEG extracts or synthetic PBAN [73,91,102]. All of the above findings indicated that PBAN might act on a target other than the pheromone gland.

One way to gain a better insight into the above issues and to resolve some of the above contradictions would be by a direct demonstration of the presence of PBAN receptors on the pheromone gland cells. We therefore synthesized two biotinilated photo-affinity (benzophenon substituted) PBAN ligands: a full-length PBAN1-33NH_2 molecule and a shorter fragment derived from its C-terminus Arg^{27}-PBAN28-33NH_2 and used them in a histochemical study to demonstrate the presence of the PBAN receptor in pheromone gland cells of *H. peltigera* females and to determine its spatial distribution in the ovipositor [12].

In order to detect PBAN receptor-containing cells among the pheromone-producing glandular cells in the ISM and other regions of the ovipositor it was necessary to perform a detailed histological study of the structure of the pheromone gland of *H. peltigera*. Although Noctuidae species have been examined more than any other family and considerable information has accumulated concerning the structure of the cells that form the sex pheromone gland, the extent to which the ISM is glandular is extremely variable among moths and there was a need, therefore, to check the actual structure of our specific moth.

We found that the pheromone gland of the *H. peltigera* female is a simple unicellular ring gland, whose glandular cells encircle the ovipositor and occupy the entire ISM between the eighth and the ninth abdominal segments [12]. Glandular cells were also found in the dorsal and ventral parts of the ninth abdominal segment, laterally divided by a sclerotized cuticular wall. By analogy with findings in other *Heliothinae* species [68,92], we assume that the cells underneath the sclerotized cuticle are unmodified squamous cells or cells modified for the insertion of muscles. Based on the above it seems that the structure of the pheromone gland of *H. peltigera* is similar to that of the glands of two other *Heliothinae* species: *H. zea*, that was found to possess ring glands with dorsal and ventral glandular cells in the ninth segment [14,48,68,82], and *H. phloxiphaga*, [48].

When both ligands were used on slide-mounted fixed sections the columnar epithelial cells throughout the ISM were stained as well as the ventral and dorsal epithelial cells in the ninth abdominal segment. Epithelial cells underlying the sclerotized cuticle were not stained. The patterns obtained with both ligands: BpaPBAN1-33NH_2 and BpaArg^{27}-PBAN28-33NH_2 were similar, indicating two possibilities: either that both ligands bind to the same receptor under the tested conditions or, if there are two distinct receptors, that their spatial distribution through out the gland is very similar. The staining exhibited a polar pattern, with intense staining appearing at the basal part of the epithelial cells. This polarity of the PBAN receptor most likely facilitates efficient contact with the hemolymph and the blood-borne hormones (e.g., PBAN) that stimulate sex pheromone production in these cells. Staining with both ligands was highly specific: no other cells in the tissue were stained, binding was fully displaced with an excess of non-biotinilated ligands (PBAN1-33NH_2 and PBAN28-33NH_2), and was not displaced with the C-terminal free acid analog PBAN1-33COOH, which is devoid of pheromonotropic activity [3]. A detailed summary of the histochemical study is described by Altstein et al. [12]. Our data clearly demonstrated the presence of a PBAN receptor on the pheromone gland, strengthening the notion that the gland serves as a target organ for the neuropeptide. Our data also reinforce previous hypotheses, based on morphological criteria, that the columnar cells are pheromone-producing cells, and correlate well with the recent data of Raina et al. [82] who demonstrated that about 70% of the pheromone-producing cells are located in the ISM.

In parallel, we have developed a binding radio-receptor assay [7,10]. We synthesized a radio-ligand (^{3}H-tyrosyl-PBAN28-33NH_2), developed a method for obtaining an active receptor preparation from the pheromone gland of the moth *H. peltigera*, and determined the optimal conditions for receptor-ligand binding with respect to membrane preparation and incubation conditions (e.g., buffers at different pH values, divalent ions and protease inhibitors). Once the assay had been optimized it was used for the characterization of the biological and pharmacological properties of the *H. peltigera* PBAN receptor in the pheromone gland.

Biological characterization involved determination of receptor expression as a function of days post-emergence, at different hours during photophase and scotophase and in different moth species (*H. peltigera*, *Helicoverpa armigera* and *S. littoralis*). Pharmacological characterization involved affinity analysis of various PBAN derived peptides and analogs. Studies in this part tested the ability of a variety of peptides derived from the PBAN sequence (PBAN9-33NH_2, PBAN13-33NH_2, PBAN19-33NH_2, PBAN26-33NH_2 and PBAN9-18COOH), linear peptides with antagonistic activity (Arg^{27}-PBAN 28-33(D-Phe^{30})NH_2, see Section 2.5 and [100]), and backbone cyclic peptides [8] (BBC 25: $n = 4$, $m = 2$; and BBC 27: $n = 4$, $m = 4$; for BBC structure please refer to Fig. 1) to compete with the radio-ligand binding to the receptor. PBAN28-33NH_2 and PBAN1-33NH_2 served as reference peptides and Arg^8-Vasotocin as a control unrelated peptide to demonstrate the binding specificity of the PK/PBAN family. We found [10] that peptides PBAN1-33NH_2, PBAN9-33NH_2, PBAN19-33NH_2 and PBAN28-33NH_2 displaced the radio-ligand at comparable potencies; PBAN26-33NH_2 was slightly less potent, and PBAN13-33NH_2 was the weakest among the agonistic linear amidated peptides. The displacement curves obtained with PBAN1-33NH_2 and PBAN28-33NH_2 were identical, with a K_i value of 3×10^{-6} M [7]. A nice correlation was obtained between the binding affinities of the tested peptides (i.e., PBAN9-33NH_2, PBAN19-33NH_2 and PBAN28-33NH_2) and their bioactivities as indicated by the in vivo pheromonotropic bioassay [2,3]. This correlation supports the notion that the receptor that was characterized in the present study is the putative PBAN receptor. Another

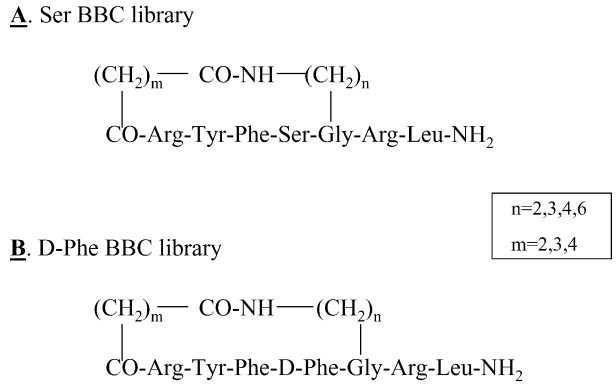


Fig. 1. General structure of backbone cyclic (BBC) peptides.

peptide that exhibited correlation was PBAN9-18COOH. The peptide did not displace the ligand up to 10 μM but stimulated binding at higher concentrations. A previous examination of the in vivo pheromonotropic activity of this peptide revealed synergism with PBAN1-33NH_2 (Altstein, unpublished). The only peptide that did not exhibit a good correlation in both assays was PBAN13-33NH_2. This peptide exhibited a much lower affinity toward the receptor in the RRA (as compared with the other linear agonistic peptides) while its in vivo pheromonotropic activity was slightly higher than that of PBAN1-33NH_2 and PBAN28-33NH_2. The reason for this mismatch is not clear at present. Arg^8-Vasotocin did not displace the ligand, indicating the specificity of the assay to PBAN-related peptides.

Interestingly, the two antagonistic peptides Arg^{27}-PBAN28-33(D-Phe^{30})NH_2 [100] and BBC peptide 25 (n = 4, m = 2) [8] had relatively low displacing activities, despite of their high antagonistic potency; furthermore, their affinity did not differ from that of the BBC peptide 27 (n = 4, m = 4), which is devoid of any agonistic or antagonistic activity. The data hint at the possibility that these antagonists are not competitive, i.e., that they do not bind to the same site as the ligand but exhibit their inhibitory activity by means of allosteric effects. Thus, in the case of the BBC peptides, it is probable that both BBC 25 (n = 4, m = 2) and BBC 27 (n = 4, m = 4) bind to the "receptor vicinity", but that only BBC 25 (n = 4, m = 2) has an allosteric effect that may inhibit ligand binding. A detailed summary of the above results has been presented by Altstein et al. [7,10].

Binding of a photoaffinity ligand to a 50 kDa protein of a crude membrane preparation of the pheromone gland was also demonstrated in *H. armigera* [76] and recently the cDNA encoding the PBAN receptor of *H. zea* has been cloned. [18]. The receptor was found to be a G-protein coupled receptor (GPCR) bearing homology to neuromedin U receptors in vertebrates.

2.4. Generation of receptor-selective PK/PBAN antagonists

2.4.1. Outline of the general approach

In the past few years, we have worked out an integrated approach, based in part on rational design, for the development of PK/PBAN antagonists based on an agonist. The approach had to be worked out almost from scratch as no methodology is available for the conversion of an agonist to an antagonist and although some vertebrate neuropeptide antagonists do exist most of them were discovered by serendipity.

Conversion of an agonist into an antagonist involves:

A. Disclosure of a lead antagonist
B. Optimization of the lead antagonists to generate a more efficient and highly potent compound

The actual steps in the conversion of an agonist into a lead antagonist involves:

A.1. Identification of the minimal sequence that forms the active site of the agonistic neuropeptide
A.2. Modification of the active sequence identified in A.1.
A.3. Determination of the SAR of the peptides that result from A.2.
A.4. Identification of a linear lead antagonist (among the modified peptides examined in step A.3.)

The rationale behind these steps is based in part on practices that have been developed in the field of bio-medical research and in the pharmaceutical industry, where attempts have been made to convert vertebrate neuropeptide agonists to antagonists. Many vertebrate neuropeptide antagonists were discovered by simple modifications of their primary sequences [21–24,29,38,43,44,57,59,69,83–87,96,98]. In order to minimize the number of possible combinations to be examined, and hence, the number of peptides to be further tested for bioactivity, it is necessary to find the shortest possible active sequence in the native neuropeptide. Once this minimal active sequence is known, sequential modifications (mainly based on substitution by D-Phe or D-Trp) are made to it, and the resulting small linear libraries are tested for bioactivity.

Once a linear lead antagonist has been made available it is necessary to improve its characteristics further in accordance with its intended applications, which means, in our case, to achieve a metabolically stable and receptor-selective antagonist. Linear peptides cannot serve such a purpose because of their high susceptibility to proteolytic degradation, their low bioavailability and their lack of selectivity (which results from their high conformational flexibility). An effective approach to overcoming these limitations is through the introduction of conformational constraint into the linear lead peptides. This leads to slower equilibrium rate, which reduces the flexibility of the molecule.

Conformational constraint can be imposed by various methods (for reviews see [37,41,42]). Cyclization of peptides is one of the commonest and most attractive methods to introduce conformational constraint into peptides and thus to restrict their conformational space [51]. The conformational constraint confers on the peptides: (i) high selectivity, by restriction of the conformational space to a conformation which mediates one function of the peptide and excludes those that mediate other functions; (ii) enhanced metabolic stability, by excluding the conformations which are recognized by degrading enzymes and thus preventing enzymatic degradation; (iii) increased biological activity, because of the much slower equilibrium between the conformations; (iv) improved bioavailability, because of the reduction of polarity. However, this is only true when the conformational space of the cyclic peptide overlaps with the bioactive conformation.

Based on the above, conversion of a linear lead antagonist into a selective, metabolically stable compound involves:

B.1. Conversion of the linear peptides to a conformationally constrained molecule

B.2. Determination of the SAR of the molecules obtained in B.1.
B.3. Discovery of a conformationally constrained antagonist (on the basis of B.2.)

Once such a molecule is available its bioactivity, bioavailability and stability must be evaluated in vivo.

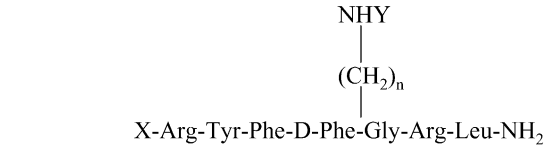
Successful application of the above strategy for the design of an optimal antagonist requires fulfillment of several preliminary requirements:

I. Knowledge of the primary sequence of the target neuropeptide (essential for step A.1. above)
II. Availability of an advanced chemistry facility for synthesis of linear, and conformationally constrained combinatorial libraries (needed for steps A.2. and B.1.)
III. Availability of in vivo or in vitro bioassays for screening the libraries and for selection of the most potent compounds (essential for steps A.3. and B.2.)
IV. Availability of a technology to impose conformational constraint on peptides (required for step B.1.)

2.5. Implementation of the strategy for the PK/PBAN family

In the past few years we have implemented the above strategy to the PK/PBAN family. The first stage involved optimization of the in vivo pheromonotropic bioassay (using female *H. peltigera* moths) for evaluation of agonistic and/or antagonistic activities of linear and conformationally constrained peptides [3,33]. Once the assay was available we synthesized a variety of linear peptides derived from the sequence of Hez-PBAN1-33NH_2, and identified (by SAR studies) the minimal active sequence of PBAN (YFSPRL-amide) that constitutes the active core of the PK/PBAN family [2,3,5,6]. Based on the hexapeptide's active sequence, a "biased library" of linear peptides was synthesized, in which each amino acid was sequentially substituted with the amino acid D-Phe. The peptides in the library were tested for their agonistic and antagonistic pheromonotropic activity (using the full-length Hez-PBAN1-33NH_2) as a stimulator, and a highly potent antagonist (RYFdFPRL-amide), capable of inhibiting sex pheromone biosynthesis by 80% (at 100 pmol) was discovered [9,100].

The sequence of the parent agonist and of the lead antagonist were used as a basis for the design of two conformationally constrained chemical libraries. Conformational constraint was imposed on the molecules by backbone cyclization [34,51]. Two backbone cyclic (BBC) libraries were designed [8]. The first (termed the Ser sub-library) was based on a slight modification of the C-terminal hexapeptide sequence (RYFSPRL-amide) of PBAN1-33NH_2, which was found to be the PBAN active core. The second (termed the D-Phe sub-library) was based on the sequence of the lead antagonist: RYFdFPRL-amide. All the cyclic peptides in each sub-library had the same primary sequence and the same location of the ring. The members of each sub-library differed in their bridge sizes and in the position of the amide bond along the bridge (Fig. 1). This part of the study was carried out in collaboration with the laboratory of Prof. Gilon at the Hebrew University of Jerusalem, who had developed the BBC methodology and the cycloscan concept [34,35].



X	Y	n
Ac	Ac	2
Ac	Ac	6
OC-$(CH_2)_3$-COOH	H	2
OC-$(CH_2)_2$-COOH	H	6

Fig. 2. General structure of the precyclic peptides.

Screening of the two sub-libraries for pheromonotropic antagonists revealed that all the antagonistic peptides originated from the D-Phe sub-library and led to the discovery of four compounds that fully inhibited (at 1 nmol) sex pheromone biosynthesis (evoked by 1 pmol PBAN1-33NH_2) and were devoid of agonistic activity (BBC 20, 22, 25 and 28; $n + m = 2 + 3$; 3 + 2; 4 + 2; 6 + 2; respectively, see Fig. 1). Substitution of the D-Phe amino acid with a Ser resulted in a loss of antagonistic activity [8,101]. Four precyclic peptides (Fig. 2), based on two of the BBC antagonists (BBC 20 and BBC 28; $n + m = 2 + 3$; 6 + 2; respectively), were also synthesized; their activity revealed that a negative charge at the N-terminus of the peptide eliminated the antagonistic activity [101]. Assessment of the metabolic stability of the BBC peptides indicated that they were very stable compared with their linear parent molecules [8,11]. To the best of our knowledge these compounds are the only known PBAN antagonists.

Evaluation of the ability of the BBC peptides to inhibit sex pheromone biosynthesis that was elicited by endogenous factors, i.e., by the natural peptides, revealed four antagonistic highly potent BBC peptides (BBC 20, 23, 25 and 28; $n + m = 2 + 3$; 3 + 3; 4 + 2; 6 + 2; respectively, see Fig. 1), that inhibited sex pheromone biosynthesis by 68, 57, 54 and 70%, respectively, for 5 h post-injection, when applied at 1 nmol [13]. Further examination of the time response of the most potent antagonist (BBC 28; $n + m = 6 + 2$) indicated that significant inhibition of sex pheromone production in *H. peltigera* females could last up to 11 h post-injection [13], indicating the high potency and metabolic stability of this antagonist. Despite the high and long-lasting potency of the BBC peptides, sex pheromone biosynthesis was not fully inhibited, suggesting that the endogenous mechanism of sex pheromone production may be either mediated by more than one receptor, or controlled by more than one neuropeptide or by more than one mechanism all of which are not affected by the above antagonists.

The availability of linear, BBC and precyclic peptides, and of the information on their pheromonotropic agonistic

and antagonistic properties under conditions in which sex pheromone biosynthesis was induced by the full-length PBAN1-33NH_2, enabled us to move toward the further characterization of the pheromonotropic antagonistic properties of the above compound under conditions in which sex pheromone biosynthesis is elicited by other PK/PBAN peptides. Three peptides of the PK/PBAN family: Pss-PT, Lem-PK (LPK, see Table 1) and Lom-MT-II (MT, see Table 1) and the PBAN derived peptide PBAN28-33NH_2 were used as stimulators. This part of the study was carried out in collaboration with the laboratory of Dr. Ronald J. Nachman at the US Department of Agriculture, College Station, TX USA. The data revealed that none of the tested compounds exhibited significant inhibitory activity (greater than 50%) toward any of the stimulating peptides (Altstein et al., in preparation). The only peptide that was slightly inhibited by two BBC peptides and a precyclic peptide was LPK, but even this inhibition was much lower that that obtained with PBAN1-33NH_2. The above results hint at the possibility that PBAN1-33NH_2 exerts its activity via a mechanism other than that used by Pss-PT, LPK, MT and PBAN28-33NH_2. Such differences may result either from the presence of different receptors (or receptor sub-types) for the different peptides on the pheromone gland, or from differences in the modes of binding of the various peptides to the receptor's active site, namely, in a manner that is not affected by the inhibitor. The later possibility can be considered only if the inhibitory compounds are non-competitive and cause inhibition through steric hindrance, which affects one ligand (PBAN1-33NH_2) but not the others (Pss-PT, MT and LPK). Confirmation of the non-competitive inhibitory nature of the BBC compounds was found in our receptor binding studies described above [10]. The reason for the different binding modes of each peptide can be explained on the basis of their size differences. PBAN1-33NH_2 is a much larger peptide (33 amino acids) than the other members of the family, so it may occupy a much larger space at the active site than the other peptides, and may thus be much more affected than the smaller peptides by steric hindrance caused by the BBC peptides. A definite answer to all of the above questions will depend on further evaluation based on SAR analysis by means of 3-D modeling of cloned receptor(s) and evaluation of their interactions with the various ligands and inhibitors. These issues are currently under investigation in our laboratory.

3. Concluding remarks and future prospects

Despite the intensive studies of the pheromonotropic activity of the PK/PBAN peptides which provided most interesting information on the chemical and molecular nature of the peptides, their origin, localization, target organ, route of transport, etc., many aspects, mainly those of their mode of action are still unresolved and currently, much remains to be learned about the structural, chemical and cellular basis of their activity, downstream cellular events, species specificity, receptor heterogeneity and functional diversity.

The information that has already been accumulated and the tools (e.g., bioassays, in vitro binding assays, receptor-selective agonists and antagonists, cloned genes, etc.,) that are currently available to us carry a high potential for further exploration of the above issue. Receptors are most significant in the understanding of the biological function of any neuropeptide (especially in families where several peptides exhibit similar bio-activities) and central in providing information on the direct correlation between the activity of a given neuropeptide and its target. Antagonists, especially those that are receptor-selective (like the BBC peptides described above) provide excellent research tools for studying multi-peptide families that exhibit functional diversity. We anticipate that the availability of conformationally constrained antagonists, the high affinity ligands that were developed in our as well in other laboratories, the cloned receptor and the binding assays that were worked out together with the in vivo bioassays provide a solid basis for further studies aiming at getting a better insight into the mode of action of PBAN and the other pheromonotropic peptides in moths. Beyond the high scientific value of the above findings the strategies and approaches that were developed in the course of the PBAN research bear also a high potential for practical application by providing a basis for generation of insect neuropeptide antagonist based insect control agents. This approach is currently being applied in our laboratory using the PK/PBAN peptides as a basis for insect control agent rational design. A detailed description of the approach has recently been described by Altstein [11,13].

Acknowledgements

This research was supported by the Israel Ministry of Science and Technology, by the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities and by US–Israel Binational Agricultural Research and Development Fund. The histochemical part of the present study was carried out while the author was on a sabbatical in the laboratory of Prof. M. Martins-Green, Department of Cell Biology and Neurosciences, University of California, Riverside (UCR) and was supported in part by the office of the vice-chancellor for research at UCR. We would like to thank Mrs. Orna Ben-Aziz and Michael Davidovitch for excellent experimental work and for insect rearing, Prof. Gilon of the Department of Organic Chemistry at the Hebrew University of Jerusalem, Israel for the design of the photo-affinity ligands and all other linear and backbone cyclic peptides, Irina Zeltser and Kalpana Bhargava currently at the Department of Chemistry, University of North Carolina at Chapel Hill, NC, USA for their synthesis and Dr. Ronald J. Nachman at the US Department of Agriculture, College Station, TX USA, for supplying LPK and MT.

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