Rational Design of Insect Control Agents: The PK/PBAN Family as a Study Case*

Miriam Altstein and Aliza Hariton

1 Introduction

The success of modern agriculture in developing and maintaining high-yield crops depends strongly on controlling insect pests by means of heavy use of insecticides, and at present organo-synthetic chemical insecticides remain the main weapon in this armory. However, in recent decades, uncontrolled application of chemical insecticides has led to acquisition of resistance by insects, has contaminated the environment with toxic residues that endanger humans and other life forms, and has disrupted the ecological balance in and around cultivated fields. The growing concern regarding the toxic effects of insecticides has led to the implementation of strict regulations in the Western World, and these are being adopted by other countries too. These regulations limit the application of some organo-chemical insecticides and ban continued use of the more toxic ones.

The strategic approach that characterizes worldwide R&D efforts is based on identification and development of novel families of non-toxic, insect-specific compounds that eventually could replace the organo-synthetic chemicals. In seeking a new class of non-toxic insecticides that eventually could replace the existing toxic organo-chemical compounds and overcome the limitations associated with the existing bio-insecticides, entomological studies are focused on the search for targets and compounds that could form a basis for the development of highly effective, selective and environmentally friendly insect control agents and/or insecticides, and emerge as a new mainstream product of the insecticide industry.

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1.1 Insect Neuropeptides as Control Agents/Insecticides

Insect neuropeptides (Nps) are prime targets in the search for novel insecticides, since they regulate many physiological and behavioral processes during development, reproduction and senescence of insects. Their blockers (antagonists) may disrupt and interfere with the normal growth, development and behavior of insects and can yield, therefore, receptor-selective, insect-specific insecticides. Such antagonists would be derived from and resemble the natural peptides, but would have to be of a peptidomimetic nature. The chemical nature of Nps enables them to be used as the basis for the design of a generic group of insect-specific and non-toxic insecticides—an 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.

1.2 Insect Nps: Historic Perspective

Since the early 1980s, many insect Nps have been identified, the basic principles of their action (e.g., biosynthesis, processing, release, transport, activation of the target cell, end degradation) have been discovered, and their roles in the physiology of organisms have been determined 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. For review see (Altstein and Nüssel 2007; Gäde and Marco 2006; Hauser et al. 2006; Hewes and Taghert 2001; Nassel and Hornberg 2006).

The first insect Nps were identified (prior to the genomic/proteomic era) by the classical techniques of isolation and Edman degradation sequencing, and their putative functions were tested in a variety of in vivo and in vitro bioassays. This approach was applied mainly to functionally identified Nps. Later, Np primary sequences were predicted on the basis of their cloned genes. In parallel, localization studies were carried out using immunohistochemical and in situ hybridization techniques and Np receptors, obtained from fractionated cell membranes were subjected to specific binding assays. These studies led to the discovery of a few tens of Nps and less than live receptors before the late 1990.

In the past few years, several major advances in research on insect Nps have enhanced our familiarization with their structures and functions. One of the most important of these advances was the sequencing of several insect genomes (Drosophila melanogaster, Apis mellifera, Bombyx mori, Anopheles gambiae and Aedes aegypti), which yielded information about Np precursor genes, on the basis of which many new Nps were identified. About 30–40 genes encoding Np precursors have been predicted in each species, and a total of over 150 insect Nps have been characterized, most of which were isolated from the fruit fly D. melanogaster, cockroaches (e.g., Lecithocheira maderae, Periplaneta americana), locusts (e.g., Locusta migratoria and Schistocerca gregaria), various kinels of
moths (e.g., *Manduca sexta*, B. mori and various Heliothinae species), and from mosquitoes (An. *gambiae* and *Ae. aegypti*). The great majority of these Nps were insect-specific.

Genome sequencing also accelerated identification of Np G-protein coupled receptors (GPCRs). Within a few years 48 Np receptor genes, which constitute the vast majority, perhaps all, of the peptide GPCRs encoded by the D. *melanogaster* genome have been predicted and, to date, nearly 30 GPCRs in this fly have been characterized with respect to their preferred peptide ligands. Peptides from other insects have also been identified, and knowledge related to *D. melanogaster* GPCRs was used to annotate 35 Np GPCRs in the recently sequenced genome of the honey bee *A. mellifera*. The genomic information also extended our knowledge regarding Np prohormone-processing enzymes and several other genes that encode proteins involved in the regulation of cell-specific Np expression. This knowledge greatly extended our potential for gaining better and deeper insight into Np functions in insects. More recently, mass spectrometry (MS) followed by sequence determination was employed for Np identification and localization. This method enabled analysis of Nps (in femtomole quantities or less) in the hemolymph, in tissue extracts and single cells by matrix-associated laser desorption/ionization (MALDI) MS, and examination of neuronal homogenates by electrospray ionization (ESI) MS. A variety of MS-based approaches (direct tissue MS or MS in combination with laser capture micro-dissection) have accelerated the development of rapid and accurate identification of predicted Nps in small amounts of tissues, and even in single neurons, and have led to both identification and localization of hundreds of new Nps in a single step.

This novel information, together with that obtained by means of more traditional approaches, opened many opportunities for the use of "rational design" approaches to the discovery of receptor-selective Np agonists and antagonists. The resulting discoveries can further advance our understanding of the mode of action of insect Nps and may serve as potential insecticide/insect management agents. Many recently published, detailed, well documented reviews of insect Nps address all the above issues (Allsteir and Nässel 2007; De Loof 2008; Gáde and Marco 2006; Hauser et al. 2006; Hewes and Taghert 2001; Hummon et al. 2006; Li et al. 2008; Nassel and Homberg 2006; Predel et al. 2007; Southey et al. 2008; Veenstra 2000).

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 then, into an insecticide or insect-control agent. Second, even when such antagonists have been made, the poor metabolic stability and the low bioavailability of these peptides – which stems partly from their difficulty in crossing membrane barriers and, especially, the 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 studies have yielded impressive progress in rational design, modification, and synthesis of chemicals, and have led to the development of several strategies for producing modified peptides and mimetic agonists and antagonists with reduced susceptibility to proteolysis and enhanced bioavailability. These new approaches, have improved the chances 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 (Höökfelt et al. 2003). Notwithstanding this progress the technology has not yet been optimized and most of the non-peptide ligands for Np receptors were found 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. For detailed reviews on conversion of Nps to drugs see (Adessi and Soto 2002: Höökfelt et al. 2003).

In the past few years, we have devised a novel integrated approach, designated Insect Np Antagonist Insecticide, 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 applied to the pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family. In this chapter we summarize the INAI approach, review the current knowledge of the PK/PBAN family of peptides, and describe the implementation of this approach to the generation of highly potent, selective and highly bioavailable PWPBAN antagonists.

2 INAI Approach to the Development of Novel Insect Np-Based Antagonist Insecticides

2.1 The INAI Approach

The INAI approach comprises two main steps:

1. Conversion of an agonist into an antagonist:
2. Conversion of the antagonist into an insect-control agent prototype.

Conversion of an agonist into an antagonist requires identification of a lead antagonist. The first steps towards this comprise:

1. Identification of the minimal sequence that constitutes the active site of the agonistic Np.
2. Modification of the active sequence identified in step 1.
Rational Design of Insect Control Agents

3. Determination of the bioactivity and structure-activity relationship (SAR) of the peptides that are generated in step 2.

3. Identification of a linear lead antagonist (among the modified peptides examined in step 3).

The rationale behind these steps derives partly from practices developed in the field of medicinal chemistry, in attempting to convert vertebrate Np agonists to antagonists. Many vertebrate Np antagonists were discovered by simple modifications of their primary sequences (Cody et al. 1995; Collins et al. 1996; Coy et al. 1989; Folkers et al. 1984; Heinzerian et al. 1987; Hruby et al. 1990, 1992; Llinares et al. 1999; Maretto et al. 1998; Piercey et al. 1981; Rees et al. 1974; Rhaleb et al. 1991; Rodriguez et al. 1987; Rosell et al. 1983; Sawyer et al. 1981; Vale et al. 1972; Vavrek and Stewart 1985). In order to minimize the number of possible combinations to be examined and, therefore, the number of peptides that require subsequent bioactivity evaluation, it is necessary to identify the shortest possible active sequence in the native Np. Once that is known, sequential modifications (mostly 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 and to optimize it in accordance with its intended applications. In the present case, this involves generating a more efficient, highly potent antagonist that is metabolically stable, and that exhibits receptor-selectivity and bioavailability. Linear peptides cannot serve such a purpose because they are highly susceptible to proteolytic degradation, they lack sufficient bioavailability, and their high conformational flexibility leads to lack of selectivity. An effective approach to overcoming these limitations is through the introduction of conformational constraint into the linear lead peptides, which leads to higher resistance to proteolytic degradation, slower equilibrium rate and reduces the flexibility of the molecule.

Conformational constraint can be imposed on peptides by various means (for reviews see Hruby et al. 1990; Giannis 1993; Goodman 1995), of which cyclization is one of the commonest and most attractive (Kessler et al. 1986). Conformational constraint: (i) imparts high selectivity by restricting the conformational space of the peptide to a conformation that mediates one function and excluding those that mediate other functions; (ii) enhances metabolic stability by excluding conformations that are recognized by degrading enzymes and thereby preventing enzymatic degradation; (iii) increases biological activity, because of the much slower equilibrium between the conformations; and (iv) improves bioavailability by reducing polarity. However, these advantages are gained only when the conformational space of the cyclic peptide overlaps the bioactive conformation.

Following the above steps, generation of an optimized antagonist involves:

5. Conversion of the linear peptide(s) to conformationally constrained molecule(s).
6. Determination of the bioactivity, SAR, selectivity, stability and bioavailability of the molecule(s) obtained in step 5.
7. Selection of a conformationally constrained antagonist with the required properties (on the basis of step 6.)

Once an improved antagonist has been identified, it must be developed into a prototype control agent. This requires the introduction of features that are normally found in commercial insecticides, i.e.: the antagonist must be converted into a low-molecular-weight compound, and able to readily penetrate through the insect cuticle and/or gut; the resulting compounds must have high environmental stability: and, especially, must be cost effective in production. The generation of small, cost-effective molecules with the above characteristics necessitates conversion of the peptide antagonist into a non-peptide small molecule (SM), and this, in turn, requires identification of the biophores that are essential for the antagonistic activity, and their incorporation into novel, scaffold SM libraries.

Biophores can be identified through detailed conformational and computational analysis of the active (agonistic or antagonistic) and inactive compounds (by means of nuclear magnetic resonance, NMR and X-Ray crystallography) (Gilon et al. 1998b; Grdadolnik et al. 1994; Kasher et al. 1999; Saulitis et al. 1992). Further SAR information can be obtained by examination of the pharmacokinetic relationships of the native and the conformationally constrained peptides with native and mutated Np receptors. This requires availability of both a cloned receptor that can be mutated, and of a functional binding assay that can be developed into a high-throughput assay (HTA) for the fast screening of the non-peptide small-molecule (SM) libraries and selection of the biologically active compounds. Once biophores have been identified, SM libraries can be generated and screened for insect control agent prototypes. This final stage involves the following steps:

8. Determination of the antagonistic conformational requirements of the peptides identified in step 7.
9. Determination of the SAR of the native and of the mutated Np receptor, and the mode of its interaction with the native and the conformationally constrained molecules.
10. Design of non-peptide SM libraries on the basis of the information gathered in steps 8 and 9.
12. High throughput screening (HTS) of the resulting SM libraries for bioactivity.
13. Identification of bioactive non-peptide SMs.

Once such non-peptide SMs have been obtained, their in vivo bioactivity, bioavailability and stability must be re-evaluated. The most potent, stable and bioavailable of them can then be subjected to appropriate formulation and preliminary toxicology evaluation and to testing in preliminary field trials. Although this strategic approach has been used in the development of some vertebrate Np agonists and antagonist-based drugs (for reviews see (Adessi and Soto 2002; Hökfelt et al. 2003), it has not so far, been used in relation to insect pest control, and still needs to be developed in this context.
An overall multidisciplinary strategy is necessary in designing an insect control agent prototype based on the INAI approach, and its success depends on the fulfillment of several preliminary requirements:

- Knowledge of the primary sequence of the target Np, on which step 1 depends.
- Availability of in vivo or in vitro bioassays for screening the libraries and for selection of the most potent compounds, on which steps 3, 6 and 13 depend.
- Knowledge and know-how in molecular design and combinatorial chemistry, on which steps 5 and 10 depend.
- Availability of or access to an advanced chemistry facility for synthesis of linear peptides and of conformationally constrained combinatorial and SM combinatorial libraries, on which steps 2, 5 and 11 depend.
- Availability of or access to specialized facilities for determination of the molecular structural conformations on which step 8 depends.
- Availability of cloned native and mutated receptors that are required for steps 9 and 12.
- Availability of a receptor-based HTS assays (HTSA) for fast screening of SM libraries and selection of the biologically active compounds, on which step 13 depends.

We applied the above strategy to the PK/PBAN family of insect Nps, and thereby identified several highly potent conformationally constrained, bioavailable antagonists, both selective and non-selective. There follows a short summary of the PK/PBAN family and the steps that led to the identification of these antagonists.

### 2.2 PBAN and PK/PBAN Family

#### 2.2.1 Isolation and Identification of PBAN and Other Pheromonotropic Peptides

PBAN was first reported by Raina and Klun (Raina and Klun 1984) as the Np that regulates sex pheromone production in female moths (*Helicoverpa zea*). In 1989 Raina et al. (Raina et al. 1989) isolated and characterized PBAN from *H. zea*, as a 33-amino-acid, C-terminally amidated Np, which was designated *Hez-PBAN* (for nomenclature see (Raina and Gade 1988)). Since 1989 the primary sequence of 19 PBAN molecules has been determined in many other moth species, by several methods: sequencing of the purified Np; from cloned cDNA; and from gene sequence or genomic data (for review see (Altstein 2004) and Table I). PBAN molecules were also found in insects belonging to orders other than Lepidoptera (e.g., in *A. gambiae* and in *A. aegypti*). Those peptides were not cloned but were predicted from the genome sequences of these insects (Holt et al. 2002; Nene et al. 2007). Comparison among the primary sequences of the above molecules revealed that the peptides share differing degrees of homology ((Jing et al. 2007; Wei et al.)
regardless of their length and degree of homology of all of them share a common C-terminal pentapeptide sequence FSPRLa.

Further studies based on molecular cloning have revealed that the sequence of the cdna encoding the PBAN pre-prohormone also encodes four other peptides, in addition to PBAN, and that they are separated by prohormone processing sequences. (single or pairs of the basic amino acids Lys or Arg, and Gly, for amidation) and that they are cleaved into four peptides which have the PBAN consensus sequence FXPRLa (X = S, T, G or V): diapause hormone, (DH), two subesophageal ganglion Nps (SGNP β and γ), and one FXPKLa peptide (α-SGNP) (see Table 1). As the number of available sequences has increased, the X in the consensus sequence has been extended to include X = I (as in B. mori and B. mandarina-β-SGNP) and K (as in D. melanogaster PK-2, see Table 1). Recent studies revealed additional variations in this family of peptides, such as the occurrence of a bioactive PBAN homolog, (in Ascoptis selenaria cretacea) that was found to be connected to β-SGNP by a GR sequence (Kawai et al. 2007). The gene that encodes these five peptides was termed the DH-PBAN gene (Sato et al. 1993; Xu et al. 1995), and it was found to be expressed only in seven pairs of neurosecretory cells, termed the DH-PBAN-producing neurosecretory cells (DHPGs) in the subesophageal ganglion (SOG) (Sato et al. 1993, 1994). Examples of the DH-PBAN peptides from a few moth species are listed in Table 1.

Advances in insect Np studies revealed that other peptides, which do not originate from the DH-PBAN prohormone, also have the same signature sequence. They include the PKs (Lcm-PK, Lorn-PK-1 and Lom-PK-11); the myotropins (Lom-MT-I to IV), which are myotropic peptides isolated from the cockroach L. maderae and the migratory locust, L. migratoria (Nachman and Holman 1991; Nachman et al. 1986; Schoofs et al. 1991. 1993b); and a myotropic peptide from S. gregaria (Seg-MT-I) (Veevaert et al. 1997). Additional peptides that were found to share the same consensus sequence are: pheromonotropin (Pss-PT) an 18-amino-acid peptide isolated from Pseudaleilia (Myrithina) separata (Matsumoto et al. 1992) that shares high homology with β-SGNP; pectitides from D. melanogaster – Drm-PK-1 (Kean et al. 2002) and Drm-PK-2 (Meng et al. 2002) – each of which is encoded by a different gene (Olsen et al. 2007); and two PK peptides from P. americana – Pea-PK-5 and Pea-PK-6 – (Predel and Eckert 2000; Predel et al. 1999). For details of the amino acid sequences of some of these peptides see Table 1. All peptides sharing the above sequences were grouped into one family, which was designated the FXPR(K)La family or the PK/PBAN family, named after PBAN and Lcm-PK which was the first member of this family to be identified, (Holman et al. 1086). and had myostimulatory ("kinin"-like) action and a pGlu residue at its N-terminus, (and thus termed pyro-kinin). Recently, a few additional peptides sharing just the PRLa C-terminal sequence were added to the family; they are not listed in Table 1 and for further information on their origins and sequences the reader is referred to Raaij and Jurenka (Raaij and Jurenka 2003).

The discovery of PBAN and the other PWPBAN peptides stimulated many studies on isolation, identification and quantification of new molecules, their gene expression and localization in various insects under various conditions, their expression during
<table>
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<th>Code name</th>
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<th>Amino acid sequence</th>
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<td>Hez-PBAN</td>
<td>Helicoverpa zea</td>
<td>LSDDMPATPADQEMYQDPEQIDSRTKYFSPR1,a</td>
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Table 1 (continued)

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<td>Hez-β-SGNP</td>
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<td>SLAYDDKSFENVETPRLa</td>
<td>(Choi et al. 1998)</td>
</tr>
<tr>
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<td>(Zhang et al. 2005)</td>
</tr>
<tr>
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<td>SLAYDDKSFENVETPRLa</td>
<td>(Duportet et al. 1999)</td>
</tr>
<tr>
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<td>Agrotis ipsilon</td>
<td>SLYEDKMFDEVETPRLa</td>
<td>(Jacquin-Joly and Burnet 1998)</td>
</tr>
<tr>
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<td>Mamestra brassicaceae</td>
<td>SLAYDDKSFENVETPRLa</td>
<td>(Iglesias and Marco 2002)</td>
</tr>
<tr>
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<td>(Iglesias and Marco 2002)</td>
</tr>
<tr>
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<td>SVAKPOTHESLEFPRLa</td>
<td>(Kawano et al. 1992)</td>
</tr>
<tr>
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<td>TMMESPRLa</td>
<td>(Ma et al. 1994)</td>
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<td>TMMESPRLa</td>
<td>(Choi et al. 1998)</td>
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<td>(Jacquin-Joly and Burnet 1998)</td>
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<td>(Iglesias and Marco 2002)</td>
</tr>
<tr>
<td>Born-γ-SGNP</td>
<td>Bombyx mori</td>
<td>TMSFSPRLa</td>
<td>(Kawano et al. 1992)</td>
</tr>
</tbody>
</table>

Bold letters indicate conserved amino acid sequences. DH - diapause hormone; MRCH - melanization hormone; PBAN - pheromone biosynthesis activating neuropeptide; PT - pheromoneotropin; PK - pyrokinin; MT - niotropin; SGNP - sub-esophageal ganglion neuropeptide.

The sequence of α-SGNP is the same in all insects (VIFTPKLa). Additional sequences of DH, u-, β-, and γ-SGNP can be found in (Jing et al. 2007).

*GenBank accession No. AB259122.

* A member of the family but with the difference that X = Lys or His.
embryonic and post-embryonic development stages, bioactivity, modes of action (e.g., cellular activity, second-messenger mediation, and their effects on the enzymatic pathway involved in sex pheromone production), and characterization of the receptor(s) that mediate their functions. These studies are reviewed in (Rafaeli and Jurenka 2003). Phylogenetic trees, based on the open reading frame (ORF) sequences of known DH-PBAN genes were also constructed and were matched with taxonomic characteristics of the insects (Jing et al. 2007; Kawai et al. 2007). More advanced studies focus on gene organization, identification of transcription factors that activate the DH-PBAN gene in different moth species (e.g., B. mori and Helicoverpa armigera) and the role of this activation in the differentiation of neuroendocrine cells (Hong et al. 2006; Shiomi et al. 2007; Zhang et al. 2004a, 2005). Earlier studies, some of which are still being evaluated, addressed transport routes, release sites, target organs, and degradation of the peptides; these are reviewed in (Altstein 2004; Rafaeli and Jurenka 2003). In the present review we briefly summarize our studies and those of other workers on a few topics in this field: (i) biological activity of the PKIPBAN family; (ii) structure-activity relationship; and (iii) characterization of the PK/PBAN receptor. Additional topics were covered in three comprehensive recent reviews (Altstein 2004; Rafaeli 2002, 2005; Rafaeli and Jurenka to which the reader is referred for further information.

2.2.2 Biological Activity of the PK/PBAN Family

The PWPBAN family of peptides is a ubiquitous multifunctional family that plays a major physiological role in regulating a wide range of developmental processes in insects: pupariation (Nachman et al. 1997); diapause (Imai et al. 1991; Nachman et al. 1993; Sun et al. 2005; Xu and Denlinger 2003; Zhang et al. 2004b; Zhao et al. 2004); cuticular melanization (Altstein et al. 1996; Matsumoto et al. 1990); feeding, i.e., gut muscle contraction (Nachman et al. 1986; Schoofs et al. 1991); and mating behavior, i.e., sex pheromone production (Raina and Klun 1984; Altstein 2004).

The first function that was discovered to be mediated by a member of the PW PBAN family – a function that also provided the name of one of its peptides, PBAN – was stimulation of sex pheromone biosynthesis in female moths (Raina and Klun 1984). Now, after nearly 25 years of research, it is well established that PBAN and other peptides of the FXPR/KLa family regulate sex pheromone production in many moth species. PBAN stimulates sex pheromone production in insects that synthesize type I pheromones (which are composed of unsaturated primary alcohols with a straight C10–C18 chain and their functional derivatives are produced from acetyl-CoA by de novo synthesis in the pheromone gland), and type II pheromones (which are composed of polyalkenes with a C17–C23 chain and their epoxy derivatives use linolenic and linoleic acids that originate in plants transported to the pheromone gland via the hemolymph after association with lipophorin, and only the epoxidation proceeds in the pheromone gland) (Kawai et al. 2007).

As studies on the bioactivity of the PK/PBAN family of peptides progressed, it became apparent that the main roles of this group of Nps are in development
processes, such as metamorphosis and diapause. Studies since the late 1990s have found that members of the family induce embryonic diapause. terminate pupal diapause, accelerate pupariation and regulate the final stage in molting, i.e., cuticular melanization. Studies on the role of the FXPR/KA peptides have indicated that they induce embryonic diapause in B. mori, through a mechanism that is yet not fully understood; it could be that the peptides act on the developing oocytes during pupal-adult development, and thereby induce diapause in the next generation (Yamashita 1996); or it could be that the hormone targets the developing ovaries, either directly, or indirectly through the DH receptors of the prothoracic gland (PG) during the later stages of the 5th instar larva and the early pupal stages, thereby regulating production of ecdysteroid hormones that might influence post-embryonic development (Watanabe et al. 2007).

These peptides are also involved in termination of pupal diapause, through their activation of ecdysteroidogenesis in the PG, in the course of larval-pupal development (Zhang et al. 2004b). Whether this activation is direct or indirect, e.g., via another factor, is also unknown so far. Recent studies suggest that DH might stimulate the PG by up-regulating expression of a DBI/ACBP gene and thereby causing an increase of ecdysteroids, which would result in diapause termination (Liu et al. 2005). Termination of pupal diapause has been demonstrated in a few Helicoverpa/Heliolitis species (H. virescens (Xu and Denlinger 2003), H. armigera (Zhang et al. 2004c) and H. assulta (Zhao et al. 2004), PWPBAN Nps also accelerated pupariation in wandering larvae of the fleshfly (Sarcophaga bullata). Studies of S. bullata revealed that Lem-PK accelerated the switch from wandering behavior to immobilization/retraction behavior, and subsequently affected puparial tanning. By accelerating both aspects of puparium formation, Lem-PK mimicked the effects of the pupariation factors, in exerting an effect on the central motor neurons (Nachman et al. 1997).

PWPBAN peptides have been reported to be involved in cuticular melanization – the last step in the molting processes – in many noctuid moths. The possible involvement of this family of Nps in the control of larval cuticular melanization was first observed in the common army worm, Leucania separata, by Ogura and co-workers (Ogura 1975; Ogura and Saito 1972; Suzuki et al. 1976). The hormone, which was termed melanization and reddish coloration hormone (MRCH), was later found to be identical with Bom-PBAN, which initiates the melanization of the integument of many moth larvae, (Ben-Aziz et al. 2005, 2006; Hiruma et al. 1984; Matsumoto et al. 1981; Morita et al. 1988).

PRAN neurosecretory cells, which express all live DH-PBAN gene peptides, were recently associated with another developmental function: development and maturation of the light motor system of holometabolous insects. Studies of B. mori revealed correlation between the rhythmic electrical activity of immature dorsal longitudinal flight muscles, on the one hand, and the bursting activity of PWPBAN neurosecretory cells, on the other hand, which suggested that the development and maturation of flight muscles during pupal-adult development is regulated by PW PBAN peptides (Kamimoto et al. 2006). Most studies of the various functions of this peptide family were carried out on moths, although a few were performed with other insects, e.g., gut contraction stud-
ies in cockroaches and locusts; and pupariation in flies. Studies performed in several laboratories, including ours, have shown that most functions can be stimulated by more than one member of this peptide family, and that the peptides are not species-specific; this subject is reviewed in (Gade 1997; Rafaeli 2002; Rafaeli and Jurenka 2003; Kamimoto et al. 2006; Ma et al. 1996). As the number of DH-PBAN-sequenced genes has increased, particular attention recently has been focused on the multifunctionality of peptides derived from the DH-PBAN-gene, i.e., DH, PBAN, a, β and γ-SGNP, as demonstrated in their pheromonotropic and diapause inducing (in embryos) and terminating (in pupae) activities. It has been found that all peptides derived from the DH-PBAN gene, except for a-SGNP, can activate pheromone biosynthesis and embryonic diapause; a-SGNP activates only on the former and not the latter (Ma et al. 1996; Sato et al. 1993).

The involvement of PWPBAN Nps in the above functions was demonstrated in a variety of in vivo and in vitro bioassays, e.g., pheromonotropic, melanotropic, diapause, pupariation and myotropic assays, that were developed and optimized in several laboratories (Altstein et al. 1996; Gazit et al. 1990; Holman et al. 1991; Matsumoto et al. 1990; Nachman et al. 1993, 1997; Raina and Klun 1984; Schoofs et al. 1991, 1993b; Warek et al. 1998). All these assays, apart from the myotropic one, were carried out in vivo.

2.2.3 Structure Activity Relationship of the PK/PBAN Family

Identification of the amino acid sequences of PBAN and of other members of the PWPBAN family made possible detailed SAR studies that used synthetic peptides derived from their sequences. Early studies on a variety of moth species have shown that the C-terminal region of the Np is essential for the pheromonotropic activity, and that within this region, the signature pentapeptide (FXPRLα; X = S) 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 (Altstein et al. 1993, 1995, 1996, 1997; Kochansky et al. 1997; Nachman and Holman 1991; Nagasawa et al. 1994; Raina and Kempe 1990, 1992). The N-terminal part of the molecule was much less important for the onset of pheromonotropic activity (Altstein et al. 1993; Raina and Kempe 1990; Kitamura ct al. 1989; Kuniyoshi et al. 1992b). A variety of Hez-PBAN-derived fragments in a range of doses applied to Heliothis peltigera at various times post-injection were used to demonstrate, that peptides lacking 12 or even 16 amino acids from their N-terminus were as active as the full-length PBAN, and that a C-terminal-derived hexapeptide that contained the signature sequence (YFXPRLα) was capable of stimulating a similar level of sex pheromone production to PBAN1–33NH, when its activity was analyzed after shorter post-injection intervals (Altstein et al. 1995); this indicated that the hexapeptide might be the biologically active site of the Np.

Structure-function studies were also performed on other insect Nps that contain the PBAN pentapeptide-C-terminal region, i.e., 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 (Abernathy et al. 1995; Fonagy et al. 1992; Kuniyoshi et al. 1992a; Nachman et al. 1993; Schoofs et al. 1993b).

Further evaluation of PKIPBAN SAR and conformational studies of the PK/ PBAN active core (based on NMR and activity analysis of conformationally con-
straint analogs) hinted that a β-turn constitutes the active conformation of these pepti-
des. In order to provide a more definitive evidence that the β-turn represents the active conformation for the PWPRAN Np family and to try and identify the exact type of the β-turn (I, I’ or II), a PWPBAN analog PK-Etz, incorporating an (E)-alkene trans-Pro isosteric component has recently been evaluated by us in four diverse PK/ PBAN bioassay systems. The conformationally constrained PK-Etz analog demonstrated activity equivalent to parent PWPBAN peptides of equal length in all four PW PBAN bioassays, and matched and/or approached the activity of peptides of natural length in three of them. The relatively potent agonist activity of PK-Etz provides strong evidence that a trans-Pro type I β-turn represents an important conformational aspect of the PWPBAN Nps during their interaction with receptors associated with a range of disparate PKIPBAN bioassays (Nachman et al. 2009d). A detailed description of these results is presented by R.J. Nachman in another chapter in this book.

The ability of a variety of peptides to stimulate sex pheromone production raised the question whether the pheromonotropic activity of the different peptides might be mediated by multiple PRAN receptors or receptor sub-types, or if all peptides mediate this activity by one receptor to which the C-terminal part of the PWPRAN Nps binds. The multi-functionality of the PWPBAN family in several different insect species raised similar questions as to whether the different functions are mediated by different receptors, located on different target organs, each activated by one or more of these Nps or whether all functions are mediated by the same receptor. Those and other questions led to an extensive study on the PK/PBAN receptors as indicated below.

### 2.2.4 PKIPBAN Target Organ and Receptors

A variety of techniques – biochemical, molecular, physiological, pharmacological and histochemical – were used to localize, isolate, clone and characterize the recep-
tors of this family of Nps in various insects. The great majority of the studies focused on the receptors that mediate the pheromonotropic activity. Early studies had suggested that PBAN might act on a target other than the pheromone gland (see (Altstein 2004) and references therein), but since the late 1990s it became clear, beyond any doubt, that the pheromonotropic activity of PBAN is mediated via the PKIPBAN receptor in the pheromone gland.

A histochemical study (Altstein et al. 2003) of the pheromone gland cells of H. peti
gera was the first direct indication of the presence of the PWPBAN receptors in the pheromone gland; The study was carried out using two biotinylated photo-
affinity (benzophenon substituted) PBAN ligands (a full-length PBAN1–33NH₂ molecule, BpaPBAN1–33NH₂, and a shorter fragment derived from its C-terminus, Arg²⁷-PBAN28–33NH₂, BpaPBAN28–33NH₂), and revealed the presence of the
receptor in columnar epithelial cells throughout the intersegmental membrane between, the eighth and the ninth abdominal segments, and also in the ventral and dorsal epithelial cells in the ninth abdominal segment. Staining revealed a polar pattern, with intense staining 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. A detailed summary of the histochemical study was presented by Altstein et al. (Altstein et al. 2003).

As in the case of other vertebrate and invertebrate Nps, the PKIPBAN Nps activate cellular processes via G-protein-coupled receptors (GPCRs), which are seven transmembrane domain proteins (7TM) that sense molecules outside the cell and activate internal signal transduction pathways and, ultimately, cellular responses. Many studies on insect Np GPCR cloning, including those of the PK/PBAN family were stimulated by the Drosophila Genome Project (Hcwes and Taghert 2001), and by the completion of the Drosophila genome sequence in 2000 and of the Anopheles genome sequence in 2002 (Adams et al. 2000; Holt et al. 2002). In less than 5 years the cDNA of 12 PK/PBAN receptors from six insects (mainly moths) were cloned and another five were only annotated (Table 2). Most of the receptors were cloned in light of the assumption of Hcwes and Taghert (Hcwes and Taghert 2001) that the PWPBAN receptor (PWPBAN-R) may be homologous to the mammalian neuromedin U receptor (NmU-R), since their ligands – NmU and the PWPBAN Nps – contain similar motifs at their C-terminus, i.e., FRPRNa and FSPRLa, respectively. Primers based on the consensus sequences of NmU and on sequences of genes CG8784 and CG8795, CG9918 and CG14575, which were annotated as coding for GPCRs in the Drosophila Genome Project, were designed and were successfully used to clone two D. melanogaster receptors (PK-2-1 and PK-2-2), two Anopheles receptors (PK-1 and PK-2), D. melanogaster PK-1 receptor (PK-1-R), and also a B. mori and an H. zea PRAN receptor (PBAN-R) (Table 2). A few receptors were cloned later on based on the already published PWPBAN-R sequences (from Spodoptera littoralis and H. armigera, Table 2). Receptors were cloned from pheromone glands of adult female moths, from several different larval instars and from pupal ovaries. Most receptors were expressed in Chinese hamster ovary (CHO) or Sf-9 cell lines; one was expressed in Xenopus oocytes (R. mori pupal ovary), and one (S. littoralis larvae) in NIH3T3 and human embryonic kidney cell lines (HEK293). In all of the studies, other than the B. mori pupal ovary, expression was monitored by Ca influx; in the case of the B. mori pupal ovary receptor it was monitored by voltage clamping.

The receptors were characterized with respect to their gene structure, homology with other cloned PKIPBAN receptors, tissue distribution, and ability to bind FXPRLa or PRLa peptides. Phylogenetic trees were constructed in searching for orthologous receptors. All the cloned receptors were found to be GPCRs, and the pheromone gland receptors from H. zea (Choi et al. 2003), H. virescens (Kim et al. 2008) and B. mori (Hull et al. 2004) were found to share a significant homology. However, the homology between the various PBAN-Rs deviated most at the N-terminus and, in some cases (Bom-PBAN-R), also in the C-terminus. H. mori-R
<table>
<thead>
<tr>
<th>Insect</th>
<th>Code</th>
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<th>Expression sys.</th>
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<td>(Kim et al. 3008)</td>
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CHO: Chinese hamster ovary cells; HEK293: Human embryonic kidney cells.
contained an extra amino acid sequence on its C-terminus, which was found to be responsible for the regulation of clathrin-mediated internalization of this receptor after it was challenged by PBAN during pheromone biosynthesis (Hull et al. 2004, 2005). All pheromone gland receptors responded to PBAN doses in the nanomolar range, in a dose-dependent manner, and also to peptides derived from the DH-PBAN gene products and to other FXPRLα and PRLα peptides – but at lower affinities. The pheromone gland receptor of *B. mori* was found to be tissue-specific, and it underwent significant up-regulation on the day preceding eclosion. Another *B. mori* receptor of the family was cloned from pupal ovaries during pupal-adult development (Homma et al. 2006). The receptor exhibited high affinity toward DH doses in the nanomolar range. The affinity of the receptor to other DH-PBAN-derived peptides was much lower.

We have cloned another PBAN-R from fifth-instar larval tissue of *S. littoralis* (Zheng et al. 2007). The receptor also showed high homology with the pheromone gland receptors, with differences in the N-terminal region, the second outer loop and the third inner loop. PBAN induced activation of an MAP kinase via a signaling mechanism that was protein kinase C (PKC)-dependent but Goi-independent. As in the case of the pheromone gland receptors, the larval receptor also responded to other PWPBAN peptides by MAP kinase activation.

Three PK receptors were cloned from *D. melanogaster*: *Drm-PK-1-R* (Cazzamali et al. 2005) and two *Drm-PK-2* receptors – *Drm-PK-2-1* and *Drm-PK-2-2* (Rosenkilde et al. 2003). These receptors are the first PK receptors predicted on the basis of the findings of the *Drosophila* Genome Project, based on the homology with neuromedin U, CG9918, CG8784 and CG8795, respectively. The *Drm-PK-1-R* was highly specific to Drm-PK-1; it reacted with all other FXPRLα and PRLα peptides but at much lower affinities. The other two receptors, *Drm-PK-2-1* and *Drm-PK-2-2*, exhibited high affinity, in dosages in the nanomolar range, toward *Drm-PK-2* only. Gene silencing by means of RNA-mediated interference (RNAi) techniques showed that silencing of the PK-2-1 gene killed embryos, whereas silencing of the PK-2-2 gene resulted in reduced viability of both embryos and first instar larvae (Rosenkilde et al. 2003). Two PK receptors, *Ang-PK-1-R* and *Ang-PK-2-R*, were also cloned from the malaria mosquito *A. gambiae* (Olsen et al. 2007). Similarly to the *Drosophila* receptors, the *Ang-PK-1-R* was selectively activated by *Ang-PK-1* peptides but not by *Ang-PK-2*. The *Ang-PK-2-R* was less selective and could be activated by both *Ang-PK-2* and *Ang-PK-1*, but with a somewhat lower affinity for the latter.

A few other receptors that belong to the PRLI/Va Np family (Capa-1, Capa-2 and Hug-y) and which share some homology with the FXPRLα/KLα family and are encoded by a capa prohormone and PK prohormone genes (which also encode for PK-1 and PK-2 in *Drosophila* and *Anopheles*), have also been cloned and characterized. Further information on their structure and characterization can be found in (Iversen et al. 2002; Olsen et al. 2007). Two other PBAN-Rs (from the moth *Plutella xylostella* and *H. armigera*) and one DH-R from *Orgyia thyellina* have been annotated (see Table 2).
2.3 Implementation of the INAI Strategy for the PK/PBAN Family

2.3.1 Discovery of PWPBAN Antagonists

Initially we optimized two in vivo bioassays for evaluation of agonistic and/or antagonistic activities of linear and conformationally constrained peptides: a pheromonomotropic bioassay with female H. peltigera adults, and a melanotropic bioassay with S. littoralis larvae (Altstein et al. 1995, 1996). Once the assays were available we synthesized a variety of linear peptides derived from the sequence of Hez-PBAN1-33NH₂, and used SAR techniques to identify the minimal active sequence of PBAN that constitutes the active core of the PK/PBAN molecule. The sequence that exhibited the same activity as that of the full length PBAN was YFSPRLa (Altstein et al. 1993, 1995, 1997). A “biased library” of linear peptides, based on this hexapeptide active sequence, was synthesized, with each amino acid being sequentially substituted with the amino acid D-Phe. We tested the peptides in the D-Phe substituted linear library for their agonistic and antagonistic pheromonomotropic activities by using the full length PBAN, Hez-PBAN1-33NH₂, as a stimulator, and discovered a highly potent antagonist – RYFdFPRLa – that was capable of inhibiting sex pheromone biosynthesis by 80%, at a dosage of 100 pmol (Altstein et al. 2000; Zeltser et al. 2000).

The discovery of a lead antagonist enabled us to take the next step: the search for improved selective and metabolically stable antagonists. For this we designed backbone cyclic (BBC) peptides, which exhibit conformational constraint and offer many advantages compatible with the requirements of improved antagonists (Gilon et al. 1991, 1993; Kessler et al. 1986). The minimal active sequence (that includes the consensus sequence of the PK/PBAN family YFSPRLa), and that of the lead antagonist (RYFdFPRLa) were used as a basis for the design of two conformationally constrained chemical BBC libraries (Altstein et al. 1999a). The first, the Scr sub-library, was based on a slight modification of the active sequence (RYFSPRLa). The second, the D-Phe sub-library, was based on the sequence of the lead antagonist. All the cyclic peptides in each sub-library had the same primary sequence and the same location of the ring; they 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 C. Gilon, of the Hebrew University of Jerusalem, who had developed the BBC methodology and the cycloscan concept (Gilon et al. 1991, 1993, 1998a). Screening of the two sub-libraries for pheromonomotropic antagonists revealed that all the antagonistic peptides originated from the D-Phe sub-library, and led to the discovery of four compounds that, at 1 nmol dosage, fully inhibited sex pheromone biosynthesis elicited by 1 pmol of PBANI-33NH₂, and exhibited no 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 (Altstein et al. 1999a; Zeltser et al. 2001). Four small BBC peptides, modified at their C-terminus (Fig. 2), and four precyclic peptides (Fig. 3), based on two of the
Rational Design of Insect Control Agents

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

A. Ser BBC sub-library

\[
(\text{CH}_2)_m \quad \text{CO} - \text{NH} \quad (\text{CH}_2)_n \\
\text{CO-Arg-Tyr-Phe-Ser-Gly-Arg-Leu-NH}_2
\]

B. D-Phe BBC sub-library

\[
(\text{CH}_2)_m \quad \text{CO} - \text{NH} \quad (\text{CH}_2)_n \\
\text{CO-Arg-Tyr-Phe-D-Phe-Gly-Arg-Leu-NH}_2
\]

\(n=2,3,4,6; \quad m=2,3,4\)

**Fig. 2** General structure of the "small" RRC peptides

28.1 \[
(\text{CH}_2)_m \quad \text{CO} - \text{NH} \quad (\text{CH}_2)_n \\
\text{CO-Arg-Tyr-Phe-D-Phe-Gly-Arg-NH}_2
\]

28.2 \[
(\text{CH}_2)_m \quad \text{CO} - \text{NH} \quad (\text{CH}_2)_n \\
\text{CO-Arg-Tyr-Phe-D-Phe-Gly-Arg-COOH}
\]

28.3 \[
(\text{CH}_2)_m \quad \text{CO} - \text{NH} \quad (\text{CH}_2)_n \\
\text{CO-Arg-Tyr-Phe-D-Phe-Gly-NH}_2
\]

\(n=6; \quad m=2\)

**Fig. 3** General structure of the precyclic peptides

\[
\text{NHY} \\
(\text{CH}_2)_n \\
\text{X-Arg-Tyr-Phe-D-Phe-Gly-Arg-Leu-NH}_2
\]

<table>
<thead>
<tr>
<th>Peptide</th>
<th>X</th>
<th>Y</th>
<th>n</th>
<th>m</th>
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<tbody>
<tr>
<td>20-L-1</td>
<td>Ac</td>
<td>Ac</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>28-L-1</td>
<td>Ac</td>
<td>Ac</td>
<td>6</td>
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<tr>
<td>20-L-2</td>
<td>OC-(CH(_2))(_n)COOH</td>
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<tr>
<td>28-L-2</td>
<td>OC-(CH(_2))(_n)COOH</td>
<td>H</td>
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BBC antagonists (BBC20 and BBC28; \(n + m = 2 + 3; 6 + 2\), respectively), were also synthesized; their activities revealed that a negative charge at the N-terminus of the peptide eliminated the antagonistic activity (Altstein 2004; Ben-Aziz et al. 2006; Zeltser et al. 2001). Assessment of the metabolic stability of the BBC peptides indicated that they were much more stable than their linear parent molecules (Altstein
et al. 1999a, 2001). These compounds were the first antagonists of the PWPBAN Nps and served as a basis for the design of other antagonistic peptides (R.J. Nachman et al., 2009c) of this family.

2.3.2 Determination of the Bioactivity, SAR, Selectivity, and Bioavailability of the BBC Antagonists

The availability of a library of conformationally constrained BBC peptides (which confers high receptor selectivity) in which some peptides were potent antagonists of PBAN-evoked pheromontropic activity and some were devoid of any biological activity made the BBC peptides also very powerful tools for examining the modes of action of the PKIPBAN family that exhibits a multifunctional pattern of activity, enabling identification of potential selective and non-selective antagonists for the various activities, which can further provide important information on whether the various functions regulated by the different PK/PBAN peptides are mediated by the same receptor or by different receptor subtypes, and/or more than one Np. Such information is indispensable for further design of insect control agents that are meant to interfere with the activity of this family of Nps. The high metabolic stability of the BBC peptides as well as their potentially increased biological activity and bioavailability also enabled to evaluate their SAR, bioavailability, and ability to interfere with the endogenous, i.e., native mechanism of action. The last of these examinations wits necessary because the bioassays that were used to select the antagonists were based on injection of a synthetic stimulator and elicitation of an "artificial" response which may not fully reflect the natural (in vivo) mechanism of action.

Evaluation of the ability of the BBC peptides to inhibit sex pheromone biosynthesis elicited by endogenous factors. i.e., by the natural peptides, confirmed that the BBC peptides did inhibit the endogenous mechanism. Four highly potent antagonistic BBC peptides: BBC-20, 23, 25 and 28 \((n + m = 2 + 3; \ 3 + 3; \ 3 + 2; \ 6 + 2, \ \text{respectively, see Fig. 1})\). were able to inhibit sex pheromone biosynthesis by 68\%, 57\%, 54\% and 70\%, respectively, for 5 h, following injection of a 1 nmol dosage (Altstein 2003). Further examination of the time response of the most potent antagonist (BBC-28: \(n + m = 6 + 2, \ \text{see Fig. 1}\) showed that significant inhibition of sex pheromone production in H. peltigera females could last up to 11 h (Altstein 2003), indicating the high potency and metabolic stability of this antagonist. The BBC peptides were also tested for their bioavailability by applying them topically on female moths at scotophase and examining the resulting inhibition of endogenously elicited sex pheromone production. The BBC peptides (BBC-25 and BBC-28: \(n + m = 4 + 2; \ 6 + 2, \ \text{respectively, see Fig. 1}\) exhibited high bioavailability (i.e., cuticular penetrability) and were able to inhibit pheromone production by 40–60\% and 50–67\%, respectively, similarly to their performance when injected to the insect (A. Hariton and M. Altstein, 2008 unpublished).

Further evaluation of the bioactivity of the BBC peptides and of some of their analogs. i.e., small BBC peptides and precyclic peptides, involved a series of
experiments that examined their ability to inhibit other PK/PBAN-mediated functions – cuticular melanization, pupariation and hindgut contraction – elicited by PBAN and other member of the PWPBAN family such as PT, Lom-MT-II, Lem-PK. Particular emphasis was placed on the question of whether elicitation of a given function by several different peptides and elicitation of several different functions by a given peptide were affected by the same or by different BBC peptides.

Interestingly, the experiments revealed differing patterns of inhibition by the BBC peptides when a given function was elicited by different peptides (Altstein et al. 2007). In the pheromonotropic assay. BBC peptides that inhibited pheromone biosynthesis elicited by PBAN1-33NH₃⁺ did not exhibit antagonistic activity against any other tested elicitor. In the melanotropic assay most BBC peptides inhibited more than one elicitor but only a few of the compounds inhibited all elicitors (Altstein et al. 2007). The inhibition patterns differed among the other PK/PBAN-mediated functions also, and in general there were marked differences between the BBC inhibition patterns of the pheromonotropic and the pupariation and myotropic assays (Altstein et al. 2007; Ben-Aziz et al. 2006). No BBC peptide inhibited the myotropic activity, although this activity was stimulated by Lem-PK, which was effectively inhibited by two BBC peptides – BBC-22 and BBC-23 (n + m = 3 + 2, and 3 + 3, respectively, see Fig. 1) – in the melanotropic assay. Lem-PK-elicited pheromone biosynthesis was also not inhibited by any of the BBC peptides. Major differences were also found between the BBC peptides that inhibited the pheromonotropic and melanotropic activities. Four selective antagonists (e.g., exhibiting an inhibitory activity toward only one function) were found, three of which were melanotropic selective antagonists (BBC-23, n + m = 3 + 3, BBC-28-L-1 and 20-L-1, see Figs. 1 through 3) and one pheromonotropic selective inhibitor (BBC-22, n + m = 3 + 2) (Altstein et al. 2007). Five non-selective melanotropic and pheromonotropic antagonists were found (BBC-20, 25, 28, n + m = 2 + 3, 4 + 2, and 6 + 2, respectively, 20-L-1 and 28-L-1, see Figs. 1 through 3), only one of which (BBC-25, n + m = 4 + 2) also inhibited pupariation. However, it is interesting to note that this peptide inhibited pupariation when it was elicited by the endogenous mechanism, whereas it did not affect it when it was elicited by exogenously injected LPK. This indicates that the endogenous mechanism may not be mediated by LPK. Selective agonists were also found amongst the BBC peptides. Six selective pure melanotropic agonists and one non-selective (melanotropic and pheromonotropic) pure agonistic compound were discovered (Altstein et al. 2007; Ben-Aziz et al. 2006).

The differing inhibitory and stimulatory patterns that were found in different assays indicated that the various functions may be mediated by structurally different receptors which do not equally recognize the BBC peptides. Although the selectivity may result from differences between the assays themselves. e.g., different insects, developmental stages, assay conditions. etc., it is also possible that it may indicate diversity in the binding pockets or in the ligand docking regions of the elicitor on the receptors that mediate the different functions resulting in an inability of the BBC peptides to block all of them. The differences obtained
within a given assay also suggested, that the PKIPBAN peptides might mediate their activity via different receptor subtypes, or that the various ligands might exhibit differing binding properties, with respect to docking or affinity, toward a given receptor and would, therefore, differ in their degrees of inhibition by the RBC peptides. Given that all the elicitors used in this study contained the same signature sequence – FXPRLa – which constitutes the active site, the differing inhibitory capabilities of the BBC peptides toward different elicitors in a given assay also suggest that their mode of inhibition is non-competitive (see also item 2.3.3, below).

The completion of this stage of the study was marked by the accumulation of a large amount of information on the SAR of the BBC peptides, on their endogenous bioactivity and bioavailability, and on their selective properties. The data also identified the most potent antagonists for each function, highlighted the residues that give the compounds their inhibitory properties and ability to penetrate the cuticle, and shed important light on their non-competitive nature. All of the above information together with the large amount of data gained recently on: (i) the high bioavailability of the native linear elicitors (PBAN, PT, LPK and MT) (Hariton et al. 2009a); (ii) the SAR anti high bioavailability of β2 anti β3 amino acid substituted peptides (Hariton et al. 2009b; Nachman et al. 2009a); (iii) the bioactivity and selectivity of the transPro mimic analogs Ac-YF[Etz]RLa and Ac-YF[Jo]RLa (Nachman et al. 2009b; Nachman et al. 2009d) (which contain an (E)-alkene termed "Etzkorn" abbreviated "Etz" moiety or a dihydroimidazoline moiety termed "Jones" abbreviated “Jo”, respectively); and on the bioactivity of a variety of amphiphilic and hydrophobic linear and cyclic molecules, peptidase resistant peptides and cytotoxic magic bullet analogs. (M. Altstein and R.J. Nachman, unpublished), set the basis for further structural analysis in support of the design of improved antagonists. One example of a successful implementation of our approach is the design of the novel amphiphilic linear D-Phe analogs Hex-Suc-AdFPRLa (in which a hexanoyl-(Hex) moiety linked by a succinic acid was incorporated to the N-terminus). The design of the peptide was based on our finding that substitution of the second amino acid in the consensus sequence of the PK/PBAN family (FXPRLa) by D-Phe results in conversion of an agonist (RYFSPLa) to an antagonist (RYFdFPRLa) (Zeltser et al. 2000). The analogs Hex-Suc-AdFPRLa was proven to be a potent and efficacious inhibitor of sex pheromone biosynthesis elicited by PBAN (84% at 100 pmol) and PT (54% at 100 pmol), but not by MT and LPK. The analog was also found to be a selective pure antagonist as it failed to inhibit melanization elicited by any of the natural PKIPBAN peptides and most important was shown to transmigrate isolated cuticle dissected from adult female Heliotis virescens moths to a high extent of 25–30% (130–150 pmol), representing physiologically significant quantities (Nachman et al. 2009c). Results on the bioactivity of all of the above peptides (amphiphilic D-Phe peptides, β-substituted analogs, PK-Etz, and PPK-Jo) are not detailed in this review and are summarized by R.J. Nachman in another chapter in this book.
2.3.3 PK/PBAN Receptor Cloning and Characterization

In order to gain additional SAR information studies focused also on the PK/PBAN receptor. The studies in this part pursued two parallel avenues: (i) cloning of the receptor in order to be able to mutate it and to study its SAR and pharmacokinetic properties more deeply; and (ii) development of a binding assay, which could later be converted into an high throughput assay, for screening libraries of chemical, molecular, and natural compounds in the search for small molecules that interact with the receptor, and which later might serve as insecticidelnsect-control prototypes.

In pursuing this objective we recently cloned a PK/PBAN receptor from *S. littoralis* larvae, which mediates cuticular melanization (Zheng *et al.* 2007), and evaluated the structure of its gene. The receptor exhibited high homology with the pheromone gland receptors, with differences in the N-terminal region, the second outer loop and the third inner loop. Currently, the structural mechanisms of the interactions between ligands and GPCRs are still not clear, but the data on GPCRs that have been accumulated so far suggest that the extracellular N-terminus/loops are responsible for the ligand docking/interaction (Leff 1995). It is thus possible that the major difference observed in the N-terminal region of the PBAN-R may, therefore, account for optimal conspecific ligand/receptor docking and interaction, which results in differing binding patterns of different ligands, (e.g., elicitors), to the pheromone gland and larval receptors. The TM helices that are highly conserved between different PBAN-Rs might form a precise ligand interaction pocket which is required for the FXPRLa motif-induced receptor conformational change and receptor activation.

The proposed model may explain the differences, (both within and between assays), between the inhibitory patterns of the BBC and those of the other antagonistic peptides, and may provide an insight into the nature (competitive or non-competitive) of the inhibitors. It is most likely that the common sequence of the PWPBAN elicitors (FXPRLa) docks in the highly conserved region of the receptors, whereas the other sequences of the elicitor molecule dock at different sites, which may differ among the various assays. In light of the above suggestions, we may further hypothesize that the various BBC and other antagonists bind outside the FXPRLa docking pocket, and may, therefore be considered as non-competitive inhibitors, whose inhibitory activity results, most likely, from interference with the docking of the other parts of the elicitor ligand with its receptor. Since different ligand molecules may dock in different regions (because of structural differences between the receptors), the degree of inhibition of a given elicitor imposed by a given BBC compound results in differing inhibitory potencies. We have previously suggested that the BBC inhibitors are non-competitive, in light of the results of an in vitro radioisotope-receptor binding study carried out with the native *H. peltigera* receptor (Altstein and Ben-Aziz 2001) (see below) and based on the in vivo assays described above. 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.
In parallel to cloning of the receptor, two binding assays were developed with the native pheromone gland PKIPBAN receptor of *H. peltigera*. The first was a radio-receptor assay (RRA) in which "H-tyrosyl-PBAN28-33NH, was used as a radio-lipand (Altstein and Ben-Aziz 2001; Altstein et al. 1999b); the second was a microplate binding assay in which a biotinylated PBAN (BpalPBAN1-33NH) was used as a tagged ligand (Ben Yosef et al. 2009). The microplate assay was designed to facilitate its further development into a HTS binding assay. A method for obtaining an active receptor preparation from the pheromone gland of the moth *H. peltigera* was developed, and the optimal membrane preparation and incubation conditions, (e.g., buffer pH values, divalent ions. and protease inhibitors) for receptor-lipid binding were determined. The experimental set up will be applied to the cloned receptor for the development of HTS binding assays.

In summary, the availability of the conformationally constrained selective and non-selective agonists and antagonists, and also of bioassays that have been developed for each of the above-mentioned functions has opened the way to a better understanding of the endogenous mechanisms of the PKIPBAN peptide family in moths and other insects. The knowledge gained in the course of the present study, regarding the effects of the compounds on the endogenous (native) mechanism, regarding their bioavailability (cuticular penetration) and their selectivity toward the various activities, as well as the data on the differing activity patterns of the PK/ PBAN elicitors and on the structural changes in the receptors that mediate their activities are all of major importance for the design of additional, improved (i.e., more potent, highly selective, metabolically stable and cost effective) agonistic and antagonistic compounds, which may, once they become available, be candidates for agrochemical applications. After formulation and preliminary field experiments, they could serve as prototypes in the development of a novel group of highly effective, insect-specific and environmentally friendly insecticides. Indeed, some of the above antagonists have already been used as a basis for the design and synthesis of novel and improved compounds with enhanced inhibitory potency and bioavailability. These compounds are currently being screened for their ability to stimulate or inhibit cuticular melanization in *S. littoralis* larvae and sex pheromone biosynthesis in *H. peltigera*. Results of these studies are not detailed in this review and are summarized by R.J. Nachman in another chapter in this book.

3 Concluding Remarks and Future Prospects

Intensive studies of the FXPRLa peptides have yielded very interesting information on the chemical and molecular nature of the PK/PBAN family of peptides, their origin, localization, target organ, route of transport, etc. In spite of this, however, many questions about these peptides are still unresolved, especially regarding their mode of action, and much remains to be learned about the structural, chemical and cellular basis of their activity, downstream cellular events, species specificity, receptor heterogeneity and the mechanisms that underlay their functional diversity.
The information that has already been accumulated and the tools, e.g., bioassays, in vitro binding assays, receptor-selective agonists and antagonists, receptor genes, etc., that are currently available to us and to other laboratories offer great potential for further exploration of the above issues. Receptors are most significant in understanding the biological function of any Np, especially in families where several peptides exhibit similar bio-activities, and they play a central role in providing information on the direct correlation between the activity of a given Np and its target. Antagonists, especially those that are receptor-selective, such as the BBC peptides described above, form 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 laboratory, the cloned receptor and the binding assays that were developed, together with the in vivo bioassays will provide a solid basis for further studies that aim to gain a better insight into the mode of action of PBAN and the other PK/PBAN peptides in moths and other insects. Beyond the high scientific value of the above findings, the strategies and approaches that were developed in the course of the PBAN research also offer high potential for practical application, by providing a basis for generation of insect Np antagonist-based insect control agents, as described in this chapter.

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