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Backbone cyclic pheromone biosynthesis activating neuropeptide (PBAN) antagonists: Inhibition of melanization in the moth *Spodoptera littoralis* (Insecta, Lepidoptera)☆

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ARTICLE INFO

Article history:
Received 7 March 2006
Received in revised form
2 April 2006
Accepted 4 April 2006
Published on line 26 May 2006

Keywords:
PBAN
MRCH
Cuticular melanization
Neuropeptide antagonists
Spodoptera littoralis
Insect neuropeptide

ABSTRACT

Antagonistic and agonistic activities of backbone cyclic (BBC) pheromone biosynthesis activating neuropeptide (PBAN) analogues were evaluated in an attempt to identify potent melanotropic antagonists, to gain an insight into their structure–activity relationship (SAR), and to discover molecules with selective and non-selective melanotropic and pheromonotropic properties. Eight potent melanotropic BBC antagonists and seven agonists were disclosed. SAR studies revealed that the structural requirements of the melanotropic and pheromonotropic agonists and antagonists are different. The cyclic structure of the BBC peptides was unimportant for antagonistic activity, and linearization retained their melanotropic and pheromonotropic antagonistic properties. Comparison of the antagonistic activities of the BBC and precyclic peptides with respect to both functions revealed eight selective antagonists (six that were selective melanotropic antagonists and two selective pheromonotropic antagonists) and four non-selective (melanotropic and pheromonotropic) antagonists. The selective melanotropic antagonists exhibited both, pure or mixed agonistic/antagonistic activities. The selective pheromonotropic compounds were pure antagonists. All non-selective compounds were pure antagonists. Comparison of the agonistic activities of the BBC peptides with respect to both functions revealed six selective melanotropic agonists and one non-selective agonistic compound. All compounds (whether selective or non-selective) exhibited pure agonistic activity. Discovery of the selective compounds hints at the possibility that the receptors that mediate the respective activities may have different properties.

1. Introduction

Insects display a wide variety of coloration and are capable of altering their pigmentation in response to external and internal factors by synthesizing pigments that may be located in the epidermal cells or the cuticle [31]. Pigmentation in insects (whether cuticular or epithelial) is controlled by endocrine and neuroendocrine factors. In locusts and many other grasshopper species (Acrididae), pigmentation and color polymorphism has been found to be controlled by a variety of neuroendocrine factors [39] such as the dark color-inducing neurohormone (DCIN, originally isolated from the CC of *Schistocerca gregaria* and *Locusta migratoria*) [40], and the adipokinetic hormone II of

☆ Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No. 500/06, 2006 series.

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0196-9781/$ – see front matter
doi:10.1016/j.peptides.2006.04.001

Schistocerca gregaria (Scg-AKH) and AKH of *Gryllus bimaculatus* (Grb-AKH) [41,42].

In noctuid moths cuticular pigmentation is controlled by the pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family. The first indication of the possible involvement of this family of neuropeptides in the control of larval cuticular melanization was demonstrated in the common army worm, *Leucania separata*, by Ogura and coworkers [29,30,38]. The hormone, which was termed melanization and reddish coloration hormone (MRCH), was later found to initiate the melanization of the integument of other moth larvae, such as *L. loreyi* [20], *Spodoptera litura* [24] and *Mamestra brassicae* [10]. Several MRCH peptides were partially purified from *Bombyx mori* [17–20,38], and the primary structure of one of them, MRCH-I, was elucidated in head extracts of adult insects [21]. MRCH-I was found to be an amidated peptide consisting of 33 amino acids and identical to PBAN of *B. mori* (Bom-PBAN-I) [14]. This neuropeptide was also found to share 80% homology with the primary structure of Hez-PBAN, isolated from *Helicoverpa zea* [33,35]. Both natural and synthetic Bom-PBAN-I/MRCH-I were found to induce cuticular melanization in larvae of *L. separata*, *S. litura*, and *S. littoralis* and to stimulate sex pheromone production in adults of *B. mori* and *S. litura* [21]. The c-DNA of *B. mori* PBAN/MRCH was cloned by Kawano et al. [13], as were many other peptides of this family of neuropeptides, all of which share a common C-terminal sequence of Phe-Xxx-Pro-Arg-Leu-NH_2 (X = Ser, Gly, Thr, Val) [2,32].

Cuticular melanization was also found to be induced by Hez-PBAN in *S. littoralis* larvae [6] and by other members of the PK/PBAN family including: Pss-pheromonotropin (Pss-PT, also termed Pss-MRCH), an 18-amino acid neuropeptide isolated from larval heads of *Pseudaletia* (=*Leucania*) *separate* [15,22], by a pheromonotropic melanizing peptide (PMP) isolated from *H. zea* (which bears 83% sequence homology with Pss-PT) [34], by *L. migratoria* myotropin-I and -II (Lom-MT-I and Lom-MT-II) and *Leucophaea maderae* PK (LPK) [11,16]. An unidentified factor, extracted from the nerve cord system, which differs from Bom-PBAN-I/MRCH-I or Hez-PBAN, was reported to be involved in cuticular melanization of *Manduca sexta* larvae [11]. Bursicon, a 40 kDa neurosecretory protein, has also been reported to induce melanization. The neuropeptide is produced by neurons in *M. sexta* and its presence has been detected in a variety of different insects. Bursicon triggers sclerotization and melanization of newly formed cuticles [23].

Studies performed in many laboratories including ours indicate that the PK/PBAN family (which currently comprises over 30 neuropeptides) is a multifunctional family of peptides, and that in addition to their ability to stimulate cuticular melanization in moths, these peptides mediate key functions associated with feeding (gut muscle contractions) [25,36], development (pupariation and diapause) [12,27,28] and mating behavior (sex pheromone biosynthesis) [2,35] in a variety of insects (moths, cockroaches, locusts and flies). These studies have shown that all of the above functions can be stimulated by more than one peptide, and that the peptides do not exhibit species specificity. For a detailed review see Refs. [2,9,32]. The functional diversity of the PK/PBAN family raises many questions with respect to the mechanisms of actions by which these neuropeptides elicit their effects, and to the nature of their receptors. Currently, our understanding of the modes of action of the various melanotropic peptides, the possible existence of multiple receptors for each function and/or for each neuropeptide, and their interactions with other components of the neuroendocrine or endocrine system is very limited, and requires further investigation. Antagonists, especially selective ones can shed light on some of these questions.

In the past few years we have developed a strategy for generation of antagonists on the basis of an agonistic neuropeptide. The strategy (termed insect neuropeptide antagonist, INA) [2] was applied to the PK/PBAN family and resulted in the synthesis of two backbone cyclic (BBC) conformationally constrained libraries: BBC-Ser and BBC-D-Phe. The first sub-library was based on the C-terminal hexapeptide sequence (Tyr-Phe-Ser-Pro-Arg-Leu-NH_2) of PBAN1-33NH_2, which comprises the active core of the PK/PBAN molecules [2,3,6,26], and the second on a potent linear antagonist of PBAN that was found in our previous studies to have pheromonotropic antagonistic properties [44]. Examination of the antagonistic activities of both libraries led to the discovery of several highly potent pheromonotropic antagonists that were able to inhibit effectively sex pheromone biosynthesis in *Heliothis peltigera* [1,2,4,43].

In the present study we tested the agonistic and antagonistic melanotropic activities of the Ser and D-Phe BBC libraries, as well as those of a few of their derivatives, by means of a quantitative melanotropic bioassay that was previously optimized in our laboratory with *S. littoralis* larvae [7], for the detection of melanotropic antagonists, determination of the structure–activity relationship (SAR) of their requirements, and for the discovery of selective and non-selective melanotropic and pheromonotropic compounds.

2. Materials and methods

2.1. Insects

S. littoralis larvae were kept in groups of 100–200 insects in plastic containers (40 cm × 30 cm × 20 cm). Sawdust was placed at the bottom of each container and the top was covered with cheesecloth. Larvae were fed on castor bean leaves and kept in a thermostatically regulated room at 25 ± 2 °C with a light/dark regime of 14/10 h and 60% relative humidity.

H. peltigera moths were reared on an artificial diet as described previously [8]. Pupae were sexed and females and males were placed in separate rooms with a light/dark regime of 14/10 h, at 25 ± 2 °C and 60–70% relative humidity. Adult moths were kept in screen cages and supplied with a 10% sugar solution. Moth populations were refreshed every year with males caught from the wild by means of pheromone traps, as described previously [8]. All females used in this study were 3.5–4.5 days old.