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Cloning and characterization of the pheromone biosynthesis activating neuropeptide receptor gene in *Spodoptera littoralis* larvae

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Abstract

In noctuid moths cuticular pigmentation is regulated by the pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family, which also mediates a variety of other functions in moths and other insects. Numerous studies have shown that these neuropeptides exert their functions through activation of the PBAN receptor (PBAN-R), with subsequent Ca^{2+} influx, followed by either activation of cAMP or direct activation of downstream kinases. Recently, several PBAN-Rs have been identified, all of which are from the pheromone gland of adult female moths, but evidence shows that functional PK/PBAN-Rs can also be expressed in insect larvae, where they mediate melanization and possibly other functions (e.g., diapause). Here, we identified a gene encoding a G-protein-coupled receptor from the 5th instar larval tissue of the moth *Spodoptera littoralis*. The cDNA of this gene contains an open reading frame with a length of 1050 nucleotides, which translates to a 350-amino acid, 42-kDa protein that shares 92% amino acid identity with *Helicoverpa zea* and *Helicoverpa armigera* PBAN-R, 81% with *Bombyx mori* PBAN-R and 72% with *Plutella xylostella* PBAN-R. The *S. littoralis* PBAN-R gene was stably expressed in NIH3T3 cells and transiently in HEK293 cells. We show that it mediates the dose-dependent PBAN-induced intracellular Ca^{2+} response and activation of the MAP kinase via a PKC-dependent but Gαi-independent signaling mechanism. Other PK/PBAN family peptides (pheromonotropin and a C-terminally PBAN-derived peptide $PBAN_{28-33}NH_2$) also triggered MAP kinase activation. This receptor, together with the previously cloned PBAN-R, may facilitate our understanding of the cell-specific responses and functional diversities of this diverse neuropeptide family.
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Keywords: Lepidoptera; PBAN receptor; GPCR; Moths; Melanization

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 (Raabe, 1989). Pigmentation in insects (whether cuticular or epithelial) is controlled by endocrine and neuroendocrine factors (Raabe, 1989). In noctuid moths cuticular pigmentation is controlled by the pyrokinin/pheromone biosynthesis activating neuropeptide (PK/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 et al. (Ogura, 1975; Ogura and Saito, 1972). 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* (Matsumoto et al., 1981), *Spodoptera litura* (Morita

Abbreviations: PBAN, pheromone biosynthesis activating neuropeptide; PK, pyrokinin; cAMP, cyclic adenosine monophosphate; PKC, protein kinase C; ERK, extracellular-signal-regulated kinase; MAPK, mitogen activated protein kinase; MEK, MAP kinase kinase; MRCH, melanization and reddish coloration hormone; GPCR, G-protein-coupled receptor; RACE, rapid amplification of cDNA ends; ORF, open reading frame; UTR, untranslated region; eGFP, enhanced green fluorescent protein; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; EGTA, Ethylene glycol-bis(2-aminoethylether)-*N,N,N,N*-tetra acetic acid; DMEM, dulbecco's modified eagle medium; BBC, backbone cyclic.

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et al., 1988) and *Mamestra brassicae* (Hiruma et al., 1984). Several MRCH peptides were partially purified from *Bombyx mori* (Matsumoto et al., 1981, 1984, 1986, 1988; Suzuki et al., 1976), and the primary structure of one of them, MRCH-I, was elucidated in head extracts of adult insects (Matsumoto et al., 1990). MRCH-I was found to be an amidated peptide consisting of 33 amino acids and identical to the PBAN of *B. mori* (Bom-PBAN-I) (Kitamura et al., 1989). This neuropeptide was also found to share 80% homology with the primary structure of Hez-PBAN, isolated from *Helicoverpa zea* (Raina et al., 1989; Raina and Klun, 1984). 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* (Matsumoto et al., 1990). The c-DNA of *B. mori* PBAN/MRCH was cloned by Kawano et al. (1992), as were many other peptides of this family, all of which share a common C-terminal sequence of Phe-XXX-Pro-Arg-Leu-NH_2 (X = Ser, Gly, Thr, Val) (Altstein, 2004; Rafaeli and Jurenka, 2003).

Cuticular melanization was also found to be induced by PBAN in larvae of *S. littoralis* (Altstein et al., 1996), 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*) *separata* (Matsumoto et al., 1992a,b), by a pheromonotropic melanizing peptide (PMP) isolated from *H. zea*, (which bears 83% sequence homology with Pss-PT) (Raina et al., 2003), by *L. migratoria* myotropin-I and II (Lom-MT-I and Lom-MT-II) and by *Leucophaea maderae* PK (LPK) (Hiruma et al., 1993; Matsumoto et al., 1993). An unidentified factor, extracted from the nerve cord system, and which differs from Bom-PBAN-I/MRCH-I or Hez-PBAN, was reported to be involved in cuticular melanization of *M. sexta* larvae (Hiruma et al., 1993). Bursicon, a 40-kDa neurosecretory protein, has also been reported to induce melanization. This 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 (Kaltenhauser et al., 1995).

Studies performed in many laboratories, including ours, indicate that the PK/PBAN peptide family, currently known to comprise over 30 neuropeptides, is a multi-functional family, and that in addition to their ability to stimulate cuticular melanization in moths, these peptides mediate key functions associated with feeding (gut muscle contractions) (Nachman et al., 1986; Schoofs et al., 1990), development (pupariation and diapause) (Imai et al., 1991; Nachman et al., 1993, 1997; Sun et al., 2005; Xu and Denlinger, 2003), and mating behavior (sex pheromone biosynthesis) (Altstein, 2004; Raina and Klun, 1984) 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 detailed reviews see Altstein (2004), Gade (1997) and Rafaeli and Jurenka (2003). The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides elicit their effects, and the nature of their receptors.

Previous research on PK/PBAN functions and signal transduction in pheromone gland cells indicated that the multi-functionality of the family is partially due to their acting via different signal transduction pathways within different cellular contexts. Several studies have shown that the interaction of PBAN with its receptor initiated a rapid Ca^{2+} response followed by second-messenger signaling, which varied somewhat among the species. For review see Rafaeli and Jurenka (2003). In *H. zea*, an elevated level of cAMP is an essential secondary messenger for PBAN regulation of enzymatic functions required for fatty acid synthesis. However, in *B. mori*, such a second messenger is not critical for this process, and in these insects, it is thought that an increase in Ca^{2+} directly activates downstream kinases (Rafaeli and Jurenka, 2003). Although much information is already available to us, our understanding of the cellular mode of action of the PK/PBAN peptides, and of the nature of the receptors that mediate these functions, still requires further investigation. Characterization of the PK/PBAN receptor(s) and further studies of their secondary messenger pathways can shed light on some of these issues.

Recently, the PBAN receptors (PBAN-R) from the pheromone gland of *H. zea*, and *B. mori* females have been cloned on the basis of their hypothetical homology with the mammalian neuropeptide receptor neuromedin U (NmU) and *Drosophila* neuropeptide receptors (Choi et al., 2003; Hull et al., 2004). Two other PBAN receptors of *H. armigera* and *Plutella xylostella* and a PK-1 receptor from *Drosophila* have been annotated (Cazzamali et al., 2005). All are G-protein-coupled receptors (GPCRs). *H. zea* PBAN-R is a 346-amino acid protein, whereas *B. mori* PBAN-R, which shares significant high homology with *H. zea* PBAN-R, is significantly longer on the C-terminus. The extra amino acid sequence on the C-terminus of *B. mori* PBAN-R, was found to be responsible for the regulation of clathrin-mediated internalization of this receptor after it is challenged by PBAN during pheromone biosynthesis (Hull et al., 2005).

In the present study we employed a homology-based-PCR approach to determine whether a PBAN receptor similar to those found in the pheromone gland in the adult was also present in the larvae where it might mediate the melanization and, possibly, regulate the pupal diapause effects of the PK/PBAN family during this stage of development of *S. littoralis*. Here, we report the cloning of a seven-transmembrane (TM) receptor protein from *S. littoralis* larvae, which is highly homologous to the PBAN-Rs present in the pheromone gland of other Lepidopteron species. This receptor is activated by PK/PBAN neupropeptides, leading to the stimulation of signal transduction mechanisms involving Ca^{2+} and extracellular-signal-regulated kinase (ERK) activation.

2. Materials and methods

2.1. Materials

Synthetic *H. zea* PBAN ($PBAN_{1-33}NH_2$), free-acid PBAN ($PBAN_{1-33}COOH$), C-terminally derived PBAN peptide, $PBAN_{28-33}NH_2$, and Pss-PT were synthesized with an ABI

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