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Isolation and functional characterization of the pheromone biosynthesis activating neuropeptide receptor of Chinese oak silkworm, *Antheraea pernyi*

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ABSTRACT

Insect pheromone biosynthesis activating neuropeptide (PBAN) controls the synthesis and actuating of sex pheromones of female adult. In the current examination, the full-length cDNA encoding the PBAN receptor was cloned from the pheromone gland (PG) of *Antheraea pernyi* (AntpePBANR). The AntpePBANR displayed the characteristic seven transmembrane areas of the G protein-coupled receptor (GPCR) and was closely related to the PBANR from *Bombyx mori* and *Manduca sexta* in the phylogenetic tree. The AntpePBANR expressed in mammalian cell lines were enacted by AntpePBAN in a concentration-dependent manner. AntpePBANR activation resulted in the calcium mobilization but did not activate the cAMP elevation pathway. Cells expressing AntpePBANR were profoundly responsive to Antpe-γ-SGNP (suboesophageal ganglion neuropeptides) and Antpe-DH (diapause hormone), different individuals from FXPRLamide (X = T, S or V) family in *A. pernyi*. Deletion of residues in the C-terminal hexapeptide (FSPRLamide) proved that P, R and L played the key parts in initiating the AntpePBANR, the amination to the last C terminal residues which can also likewise impact the activation of AntpePBAN receptor altogether. The mRNA of the AntpePBANR gene demonstrated the most noteworthy transcript levels in pheromone gland followed by fat body.

1. Introduction

Insect pheromone biosynthesis activating neuropeptides (PBAN) belong to FXPRL amide family of neuropeptides are known to direct amalgamation and influx of sex pheromones, and furthermore to control the following species-particular courtship and mating [1]. PBAN is predominantly originated from the suboesophageal ganglion (SG) and it can be transported toward their target tissue pheromone gland (PG) [2]. PBAN ties to its own particular receptor and exerts its consequences for cellular targets. Previous reports demonstrated that PBAN receptor possesses seven hydrophobic membrane-spanning regions and the rhodopsin-like G protein-coupled receptors (GPCRs) at high-affinity binding sites [3,4].

The first PBAN receptor was identified and characterized from *Helicoverpa zea* (Noctuidae) [3], followed by second one from *Bombyx mori* (Bombycidae) [4]. In past years, PBANRs in other 13 species from lepidopteran have been identified and characterized, including *Ostrinia nubilalis* from Crambidae [5]; *Plutella xylostella* from Plutellidae [6]; *Mamestra brassicae* [7], *Agrotis segetum* [8], *Helicoverpa armigera* [9], *Mythimna separate* [9], *Heliothis peltigera* [10], *Heliothis virescens* [11], *Spodoptera exigua* [12], and *Spodoptera littoralis* [13] from Noctuidae. The nucleotide and putative amino acid sequences of cDNA encoding PBAN receptor from *Manduca sexta* (ACQ90219, ACQ90220, ACQ90221, & ACQ90222), *Spodoptera litura* (AJW32184) and *Chilo suppressalis* (ALM88337 & ALM88338) were reported in GenBank. PBAN like peptide in insect other than the order Lepidoptera has been named as pyrokinin. The pyrokinin receptors had been identified and characterized from *Drosophila melanogaster* [14,15], *Tribolium castaneum* [16,17], *Ixodes scapularis* [18], *Rhipicephalus microplus* [19], *Rhodnius prolixus* [20] and *Anopheles gambiae* [21]. All the identified PBAN (or pyrokinin) receptors contain several characteristic features of type 1 seven TM GPCR [7,14–21]. Previous study has confirmed that these reported PBAN receptors could be activated by their corresponding PBAN in a dose-dependent manner and in response to another insect PBANs or pyrokinins [11]. Several studies have observed multiple PBANR isoforms (PBANR-As, -A, -B, and -C) due to the alternative

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splicing of the C-terminus. The "short" isoforms lack a C-terminal extension and exhibit different ligand-induced internalization as compared to the "long" PBANRs [9]. The activation of PBAN to PBANR in *H. zea* could promote the elevation of both intracellular calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) concentrations [3]; however, only the intracellular calcium (Ca^{2+}) was increased after PBAN binding to PBANR in *B. mori* [4]. The above difference demonstrating that the second messengers to transduce the PBAN signal to the target cell and the organism were different in various studied insects.

Because of the great economic interest, Chinese oak silkworm, *Antheraea pernyi* has been wildly reared as one of the most popular silkworm species particularly in China, India, and other Asia countries [22,23]. Previously, we reported the full-length cDNA encoding pheromone biosynthesis activating neuropeptide (PBAN) in *A. pernyi* which was cloned and sequenced and encoding five C-terminal pentapeptide motif FXPR/KL (X = G, T, S) peptides, including a 33-aa PBAN, a 24-aa DH-like peptide (AntpeDH), 7-aa α-SGNP (Antpeα-SGNP), 21-aa β-SGNP (Antpeβ-SGNP), and 8-aa γ-SGNP (Antpeγ-SGNP) [24]. G Protein-coupled receptor is always used as a potential target to demonstrate the function and signal transduction of specific gene [25,26]. To understand the PBAN signal in sex pheromone biosynthesis of *A. pernyi*, PBAN receptor needs to be identified. Herein, we cloned the full-length cDNA encoding PBAN receptor (AntpePBANR) from the PG of *A. pernyi* using RT-PCR and RACE methods. The AntpePBANR was expressed in heterologous cell lines and AntpePBAN and its analogs were evaluated for their role in activation of AntpePBANR. Through systematic deletion, each residue was characterized for their functional role in the active core which can interact with the specific receptor.

2. Materials and methods

2.1. Insects

In the current study, 'Qinghuang' strain of Chinese oak silkworm *A. pernyi* was used for experimentation. Larvae of Chinese oak silkworm were reared under a long-day photoperiod at L14:D10 and 25 °C ± 1 °C [24]. The 0.75% NaCl was used during the dissection and dissected tissues were stored at −70 °C until use.

2.2. RNA extraction, RT-PCR and RACE amplification

For RNA extraction, the dissected tissues (<10 mg) were grinded in a MagNA Lyser® Instrument (Roche). The tissue homogenate was target for total RNA extraction using an Eastep™ Total RNA Super Extraction Kit (Promega). The total RNA was transcribed into cDNA employing oligo dT's as described in the protocol using the PrimeScript™RT reagent Kit (TaKaRa).

Two degenerate primers ABPRF1 and ABPRR1 (Table 1) were synthesized and further used for amplification of a fragment of Anp-PBANR cDNA. The obtained PCR products was purified and sequenced. Based on the sequence for fragment of Anp-PBANR cDNA, specific primers ASF1 and ASR1 (Table 1) were designed for 3′- and 5′-RACE. The 5′- and 3′-RACE experiments were performed according the description given by Zhang et al. using SMART™ RACE cDNA Amplification Kit (Clontech) [27].

Table 1
Primers used in present study.

Name	Primer sequence	Purpose
ABPRF1	*aaccgctccatgcacaccgccac*	Primers used for amplification of fragment of AntpePBANR
ABPRR1	*ttgttcgacatdatgttgtabag*	
ASF1	*catacgacgagaacggacaga*	Primers used for RACE for AntpePBANR
ASR1	*acgtgcgcagctttacaccga*	
AnpORFF	*ctcaagcttatgaagccgaagtacgaaaaca*	Primers used for amplification of ORF of AntpePBANR
AnpORFR	*cttgatatcgccgtgtctcaggtaagtcctc*	
PRF	*tatctctttagtctcgcaatatca*	Primers used for q-PCR for AntpePBANR. Target production is 153 bp.
PRR	*tgtcgcattcgccgacgtttcagag*	
PF	*tgccagctactcccaaagat*	Primers used for q-PCR for AntpePBAN. Target production is 161 bp.
PR	*cgccactcgtacgttgattg*	
ActinF	*gcagaaggaaatcacagccc*	Primers used for q-PCR for Actin. Target production is 181 bp.
ActinR	*cttcctgtgcacgattgagg*	

2.3. Phylogenetic and structural analysis

Primer5.0 and DNAMAN (LynnonBioSoft) was used for analysis of the nucleotide sequence and deduced amino acid sequence of AntpePBANR on the basis of structural features of other PBANR sequences. Same sequences were found using BlastP in the non-redundant protein sequences (nr) database of the NCBI website (http://www.ncbi.nlm.nih.gov/). The AntpePBANR sequence was compared to other PBANR sequences. The obtained PBANR sequences of various organisms were aligned with Multiple Sequence Comparison using MEGA ver5.0 software [28] and further a phylogenetic tree was constructed via neighbor-joining method. For the analysis of constructed tree, percentages of replicate trees were displayed next to the branches. Poisson Correction method was used for calculating the evolutionary distances in the units of number of amino acid substitutions per site. All positions containing gaps and missing data were not considered in the final dataset through complete deletion [29].

2.4. Peptides synthesis

AntpePBAN, AntpeDH, Antpeα-SGNP, Antpeβ-SGNP, Antpeγ-SGNP and other mimetic peptides were synthesized by China Peptides Co., Ltd. (Shanghai). 10% acetonitrile was used for preparation of peptide stocks and dilution was carried out into Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12-Ham (DMEM-F12) supplemented with 0.1% bovine serum albumin (BSA) to obtain working solutions of the obtained peptides. The BCA Assay (Pierce) was performed to determine the peptide concentrations of the stocks.

2.5. Cell culture and transfections

The cells lines used in this study were as followed: Chinese hamster ovary (CHO) K1 and human embryonic kidney (HEK) 293 cells which were procured from Professor Park Yoonseong from Kansas State University. Prior to their direct use, they were cultured in monolayers in DMEM-F12 (Sigma-Aldrich, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 100 IU/mL of penicillin, 250 mg/mL of Zeocin (Invitrogen) and 100 μg/mL of streptomycin (Invitrogen). The cultured cells were target for their pharmacological analyses which was performed in CHO-K1 cells showing the steady expression of the promiscuous Gα16 without considering their natural signaling cascade. Further, both the cell lines were subjected for AntpePBANR downstream signals measurement via calcium and cAMP, respectively.

The ORF of AntpePBANR was amplified with the primers AntpeORFF and AntpeORFR (Table 1) and pcDNA™5 vector (Invitrogen) was inserted. Transfection with pcDNA™5-AntpePBANR or empty pcDNA™5 vector was performed in T75 flasks. The 27 μL of FuGeneHD (Invitrogen) and 900 μL of DMEM-F12 construct mixture was used for preparation of transfection medium for CHO-K1 cells. After 15 min incubation, medium was supplemented with 9 μg of pcDNA™5-AntpePBANR and 9 μg of bioluminescent protein apoaequorin (HcyAg) DNA. After 45 min incubation without direct light exposure, the medium was replaced by 25 mL of serum-free medium in a drop wise manner. The same transfection medium used for both the cell lines. Following transfection, cells were incubated overnight (37 °C, 5% CO_2) which was followed by addition of 1 mL of heat-inactivated FBS. Subsequently, ligand-induced changes in either intracellular calcium or cAMP were monitored in the cells [16,27].