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Research paper

Identification and functional characterization of the pheromone biosynthesis activating neuropeptide receptor isoforms from *Mamestra brassicae*

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ABSTRACT

In most moth species, including *Mamestra brassicae*, pheromone biosynthesis activating neuropeptide (PBAN) regulates pheromone production. Generally, PBAN acts directly on the pheromone gland (PG) cells via its specific G protein-coupled receptor (i.e. PBANR) with Ca^{2+} as a second messenger. In this study, we identified cDNAs encoding three variants (A, B and C) of the *M. brassicae* PBANR (Mambr-PBANR). The full-length coding sequences were transiently expressed in cultured *Trichoplusia ni* cells and Sf9 cells for functional characterization. All three isoforms dose-dependently mobilized extracellular Ca^{2+} in response to PBAN analogs with Mambr-PBANR-C exhibiting the greatest sensitivity. Fluorescent confocal microscopy imaging studies demonstrated binding of a rhodamine red-labeled ligand (RR10CPBAN) to all three Mambr-PBANR isoforms. RR10CPBAN binding did not trigger ligand-induced internalization in cells expressing PBANR-A, but did in cells expressing the PBANR-B and -C isoforms. Furthermore, activation of the PBANR-B and -C isoforms with the 18 amino acid Mambr-pheromonotropin resulted in co-localization with a *Drosophila melanogaster* arrestin homolog (Kurtz), whereas stimulation with an unrelated peptide had no effect. PCR-based profiling of the three transcripts revealed a basal level of expression throughout development with a dramatic increase in PG transcripts from the day of adult emergence with PBANR-C being the most abundant.

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1. Introduction

The vast majority of sexually receptive female moths produce species-specific sex pheromones to attract conspecific males (Karlson and Butenandt, 1959). These compounds are usually *de novo* synthesized via a modified fatty acid (FA) biosynthetic pathway (Rafaeli, 2002; Tillman et al., 1999). Biosynthesis and release typically takes place within the specialized epidermal tissue that comprises the pheromone gland (PG), which is located at the intersegmental membrane between the 8 and 9th abdominal segment (Blomquist and Vogt, 2003; Percy and Weatherston, 1974).

In recent years, understanding of the biosynthetic pathways underlying lepidopteran pheromone production and its regulation has been greatly expanded (Blomquist et al., 2011; Jurenka, 2004; Jurenka and Rafaeli, 2011; Matsumoto et al., 2007; Rafaeli, 2011). In mature virgin females, both pheromone production and release are synchronized by the circadian-regulated action of pheromone biosynthesis activating neuropeptide (PBAN), which originates from the subesophageal ganglion (SEG) (Raina et al., 1989). PBAN is a member of an ancient conserved signaling family of peptides characterized by a C-terminal amidated pentapeptide FXPRL-amide (X = S, T, G or V) that is essential for the pheromonotropic activity of PBAN (Kuniyoshi et al., 1991; Raina and Kempe, 1990). Post-translational processing of the PBAN prepropeptide yields PBAN as well as four additional FXPRL-amide peptides i.e. diapause hormone and α-, β- and γ-SEG neuropeptides (SGNPs) that exhibit varying degrees of pheromonotropic activity (Ma et al., 1994, 1996; Sato et al., 1993).

Pheromone production is initiated by specific binding of PBAN to its cognate receptor (PBANR) expressed at the cell surface of

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PG cells. Although demonstrated histochemically in *Heliothis peltigera* PGs (Altstein et al., 2003), the molecular identity of the PBANR proved elusive until sequence similarities between predicted Drosophila G protein-coupled receptors (GPCRs) and mammalian receptors suggested that FXPRL-amide receptors, such as PBANR, belong to the mammalian neuromedin U receptor family (Hewes and Taghert, 2001). Subsequent confirmation of this hypothesis (Park et al., 2002) facilitated homology-based identification of the PBANR in *Helicoverpa zea* (Choi et al., 2003) and *Bombyx mori* (Hull et al., 2004). Since then PBANRs have been identified in diverse moth species (Cheng et al., 2010; Ding and Löfstedt, 2015; Kim et al., 2008; Lee et al., 2012; Nusawardani et al., 2013; Rafaeli et al., 2007; Zheng et al., 2007) with multiple PBANR isoforms (PBANR-As, -A, -B, and -C) shown to be concomitantly expressed in PGs (Kim et al., 2008; Lee et al., 2012). The isoforms are differentiated by the length and composition of their C-termini, which is necessary for the ligand-induced internalization (Hull et al., 2005), a phase of GPCR feedback regulation and desensitization (Moore et al., 2007; Marchese et al., 2008). The "short" PBANRs lack a C-terminal extension and exhibit different internalization kinetics as compared to the "long" isoforms (Lee et al., 2012). The differing responses of the isoforms, which arise from alternative splicing of the C-terminus (Kim et al., 2008; Lee et al., 2012), have been suggested to reflect the pleiotropic nature of the PK/PBAN signaling system in insects (Nusawardani et al., 2013). Despite these structural differences and variation in the downstream intracellular signal transduction cascade (Hull et al., 2007a; Matsumoto et al., 1995; Rafaeli et al., 1990; Rafaeli and Soroker, 1989), PBANRs are characterized by PBAN-mediated mobilization of extracellular Ca^{2+}, which is prerequisite for turning the PBAN signal into the biological response of sex pheromone production (see reviews: Matsumoto et al., 2010; Rafaeli, 2009).

Although not as extensively studied as the two main model systems (i.e. *B. mori* and *H. zea*), *Mamestra brassicae* has also been the focus of numerous pheromonotropic-based studies. Immunocytochemical studies revealed the presence of PBAN-like peptides in the hemolymph at 100–500 pM during the scotophase (Iglesias et al., 1999) and a cDNA clone of the PBAN coding sequence with significant homology to *H. zea* PBAN (Helze-PBAN) has been reported (Jacquin-Joly et al., 1998). In addition, a pheromonotropic 18-aa SGNP, referred to as Mambr-PT (Fónagy et al., 1998, 2008), with close sequence similarity to β-SGNPs identified in *B. mori, H. assulta, H. zea,* and *Pseudalatia separata* (Altstein et al., 2013; Jurenka, 2015; Rafaeli, 2009) has been identified. More recently, an extensive study examining the time course of *M. brassicae* pheromone production in relation to a desaturase further revealed the pheromonotropic effects of Mambr-PT (Köblös et al., 2015). Despite these findings, the PBANR of *M. brassicae* remains unknown. The objective of this study was to clone and characterize PBANRs in the pest *M. brassicae* and to determine if these receptors are functionally similar to those described in other noctuid moths (Jurenka and Rafaeli, 2011). The generation of new insights into PBANR – PBAN interactions may elicit species specific control of pheromone production in pest management and provide a potentially deeper understanding to facilitate the development of new antagonists (Nachman et al., 2009).

2. Materials and methods

2.1. Insects

A laboratory culture of *M. brassicae* was established using adults collected from fields in different regions of Hungary and maintained under a reverse photoperiod of 16:8 h light/dark cycle at 25 °C, 50% relative humidity. Larvae were reared on a semi-synthetic diet (Nagy, 1970). Pupae were sexed and separated and after moth emergence, adults were kept separately in glass jars (12 × 10 cm) covered with fine mesh and fed a 10% honey solution supplied on cotton pads.

2.2. Cloning and sequence analysis

Total RNA was isolated from PGs of 2–3-day-old calling females using Extractme Total RNA kit (Blirt SA, DNA-Gdańsk, Gdańsk, Poland) and reverse transcribed using SuperScript III reverse transcriptase and random hexamer primers (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A 1208-bp fragment of *M. brassicae* PBANR (Mambr-PBANR) was amplified with degenerate primers (sense 5′-CGCCGCAACTYYGTCGTTC-3′ and antisense 5′-TRAAGGCGTTTCTGAACTTRTT-3′) corresponding to conserved regions of published PBANR sequences. PCR amplification was carried out using Phusion DNA polymerase in HF buffer (Thermo Fisher Scientific, Waltham, MA) under the following conditions: 98 °C for 1.5 min, 35 cycles at 98 °C for 15 s, 59 °C for 30 s and 72 °C for 40 s, followed by a final extension of 72 °C for 5 min. The amplified PCR products were ligated into the pJET1.2/blunt cloning vector using a CloneJET PCR cloning kit (Fermentas, Glen Burnie, MD) according to the manufacturer's protocols and sequence verified (Macrogen Europe, Amsterdam, The Netherlands).

Sequence information from the cloned cDNA fragments was used to design gene-specific primers using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Full-length cDNA sequences were determined by 5′- and 3′-rapid amplification of cDNA ends (RACE) using a SMARTer RACE cDNA amplification kit (Clontech, Palo Alto, CA) according to the recommendations of the manufacturer. The two specific primers designed from the partial cDNA fragment were 5′-GAGTGGGCAGATGCAGTACAG-3′ for 3′ RACE, and 5′-CTTGAACAGTAGACGCGGAACAT-3′ for 5′ RACE. The sequences have been deposited in GenBank under accession Nos. KX831609–KX831611.

Sequence alignments of the putative protein sequences were performed with ClustalX 2.1 (Thompson et al., 1997). Transmembrane regions of the PBANR proteins were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM) (Krogh et al., 2001). Phylogenetic analysis of the putative Mambr-PBANR sequences was performed in conjunction with other lepidopteran PBANR sequences obtained from BLAST searches. The phylogenetic evolutionary history was inferred using the Maximum-Likelihood method according to the Le-Gascuel model (Le and Gascuel, 2008) implemented in MEGA 6 (Tamura et al., 2013) with bootstrap support based on 1000 iterations.

2.3. Construction of insect expression plasmids

To examine the cellular localization of the Mambr-PBANR isoforms and ligand binding, chimeras of each isoform were constructed with the fluorescent protein mVenus (Nagai et al., 2002) fused in-frame to the receptor C-terminus. Expression vectors were constructed using a pIB/V5-His-TOPO TA insect expression vector (Thermo Fisher Scientific, Waltham, MA) containing the mVenus coding sequence engineered with a 5′-StuI restriction site (i.e. pIB/StuI-mVenus). The variants were amplified using a gene specific ORF sense primer (5′-AAGATGACATTGCCAGTGCC-3′) and variant specific antisense primers (PBANR-A: 5′-CCTCAATACAAG TAGATCAATCATA-3′; PBANR-B: 5′-CCTGGTGAGTCCGCCGAT-3′; PBANR-C: 5′-CCTCGTGGTACATGTAGGTGG-3′) that replaced the stop codon with an in-frame StuI restriction site. The amplified PCR products were then cloned into the StuI restriction site of the mVenus expression vector. The resulting constructs were sequence validated prior to use.

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