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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.

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

433A automatic peptide synthesizer on Rink amide MBHA resin by means of the FastMoc™ chemistry as described previously (Altstein et al., 2003). Chemical inhibitors of MEK 1/2 (PD98059), PKC (Gö6983) and Gαi (cholera toxin) were purchased from Calbiochem. Phosphorylated ERK antibody was purchased from Cell Signaling and ERK 2 antibody from Upstate Biotechnology. All the oligonucleotides were synthesized by Sigma-Genosys.

2.2. Insects

S. littoralis larvae were kept in groups of 100–200 insects in plastic containers measuring 40×30×20 cm. Sawdust was placed at the bottom of each container and the top was covered with cheesecloth. The 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. The larvae were collected at the 5th instar, and kept frozen at −80 °C until used.

2.3. Receptor cloning

Poly(A)+ RNA was isolated with Oligotex Direct mRNA isolation kit (Qiagen) followed by the reverse transcription reaction carried out with SMART II RACE kit (Clontech). Degenerated primer pairs, were designed based on the high-homology regions of *H. zea*, *H. armigera* and *B. mori* PBAN-R genes (Choi et al., 2003; Hull et al., 2004) and used to PCR-amplify the internal region of this gene. Primer pairs, sense 5′-ACNGCNTTYACNGTNGARCG-3′ and antisense 5′-GCRTGRAANGGNGCCCARCA-3′ successfully amplified a 420 bp fragment with the Access One-Step RT-PCR System (Promega) followed by PCR after the reverse transcription reaction under these conditions: 35 cycles at 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min. This fragment was cloned into pCR2.1-TOPO vector (Invitrogen) and subsequently sequenced. To obtain both ends of this cDNA, gene-specific primers were used (3′-RACE gene-specific primer: 5′-CAGGAATGGAG-CATCACAGA-3′, 5′-RACE gene-specific primer: 5′-CACAACGCAAACACCCAAATA-3′) in conjunction with the SMART II cDNA RACE kit (Clontech). Following the determination of the sequences of the 5′ and 3′ untranslated regions (UTRs) of this gene, available sequences were assembled and an open reading frame (ORF) of 1050 base pairs was predicted. A primer pair, sense 5′-CTAACGTGGATCTA-CAAAATT-3′ and antisense 5′-AAAATGGCGTCTTT-GAGGG-3′ was designed for PCR amplification of the entire predicted ORF of the gene, and the PCR product was subsequently cloned into pCR2.1 cloning vector for sequencing. Ten individual clones from the PCR product were fully sequenced and yielded entirely identical sequences for the ORF.

2.4. Receptor expression in cells

In the wild-type *S. littoralis* PBAN-R cDNA that we obtained, the start-codon (ATG) of the ORF was not associated with any identifiable KOZAK sequence motif (Kozak, 1986). In order to enhance the expression of this gene in mammalian cells, mutations were introduced by mismatch-PCR preceding the start-codon, creating an ideal KOZAK signal GCCACC immediately before the start-codon ATG with a partially mismatched PCR primer 5′-AAGCCACCATGACATTGT-CAGCG-3′, whereas no mutation was introduced into the ORF. This modification was subsequently confirmed by sequencing. The receptor ORF with the KOZAK signal sequence was cloned into a retroviral vector, pIB-2 (gift of Nolan GP, Stanford University) which co-expresses a blasticidin-resistant selection marker for retroviral expression of PBAN-R in NIH3T3 cells, or cloned into the pcDNA 3.1 vector (Invitrogen) for transient expression of PBAN-R in HEK293 cells. To establish stably infected NIH3T3 cells, the retrovirus was produced with the 293T packaging cell line, pseudotyped with VSV-G and used to infect murine NIH3T3 cells, with the aid of polybrene at 5 μg/ml as previously described (Li et al., 2004, 2005). Infected cells were selected with 10–15 μg/ml of blasticidin for 7–10 days. The cells that survived were expanded and used for our assays. To test the PBAN-R in a different cell type, the pcDNA-PBAN-R was transfected into HEK293 cells at 80% confluency with the MetafectenePro (Bionex) transfection reagent according to the manufacturer's instruction. This procedure usually yields a positive transfection rate >95%, as determined by co-transfection of an eGFP construct (Clontech). Transfected cells were serum-starved for 1 day and then used for various assays.

2.5. Single-cell Ca^{2+} mobilization assay

The dynamics of PBAN activation of the *S. littoralis* PBAN-R was determined by measuring changes in the intracellular Ca^{2+} concentration. Briefly, approximately 2×10^4 NIH3T3 cells expressing the *S. littoralis* PBAN-R were cultured in DMEM supplemented with 1% fetal bovine serum in 5% CO_2 atmosphere at 37 °C on poly-L-Lysine (Sigma) coated glass coverslips (Warner Instrument) before the Ca^{2+} assays. The ratiometric Ca^{2+} indicator Fura-2 acetoxymethylester (Molecular Probes) (1 μM) was freshly reconstituted in DMSO and diluted in assay buffer (145 mM NaCl, 3 mM KCl, 15 mM HEPES, 1 mM Na_2HPO_4, 6 mM Glucose, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, pH 7.4) containing 0.05 g/L pluronic F-127 to a final concentration of 2 μM. Cells were loaded with Fura-2 in the dark at room temperature for 30 min, then mounted on the stage of Nikon TE300 fluorescence microscope. Concentrated PBAN or other peptides diluted in assay buffer was carefully pipetted onto the cells into final ligand concentrations indicated on figures. The cells were started to be monitored about 3–5 min before the addition of any peptides, and the monitoring was conducted by alternative illumination of the cells at 340 and 380 nm, and the emitted light was collected by a 40× objective (Nikon S-Fluor, NA 0.90) and filtered through a 490–530 nm bandpass filter. Images were captured every 0.5 s with a cooled digital camera (Hammamatsu Orca-100) and analyzed with MetaFluor software (Molecular Devices). Intracellular Ca^{2+} concentration was estimated from the ratio of fluorescence intensities at

340 and 380 nm by means of the Grynkiewicz equation (Grynkiewicz et al., 1985). Maximal and minimal fluorescence was determined by exposing the cell to 1 μM ionomycin in the absence of, and then in the presence of 5 mM EGTA.

2.6. Immunoblots

HEK293 or NIH3T3 cells expressing PBAN-R were serum-starved in DMEM supplemented with 1% fetal bovine serum for

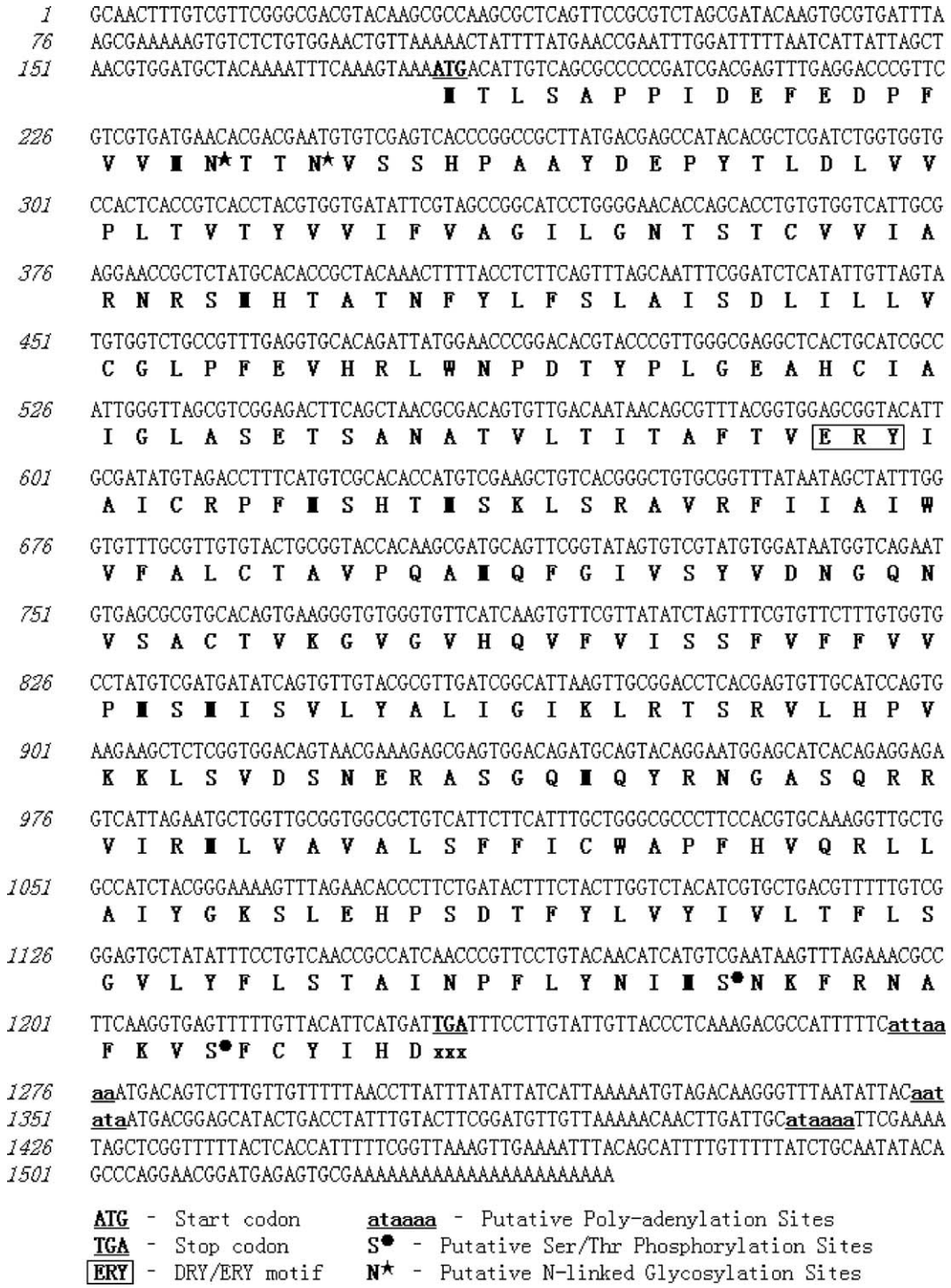

Fig. 1. Nucleotide and deduced amino acid sequence of the *S. littoralis* PBAN-R. The nucleotide sequence of the 5′- and 3′-UTR flanking the ORF was determined by 5′ and 3′ RACE. The exact sequence for the ORF was derived from 10 clones produced from PCR products obtained by using primers designed for 5′- and 3′-UTRs. The nucleotide sequence is numbered from the 5′ to the 3′ direction; key features of the gene are marked. The first ATG nucleotide sequence (underlined bold print) present in the cDNA was identified to be in frame with the predicted ORF. The ORF has a length of 1050 nucleotides followed by a TGA stop codon (underlined bold print). In the 3′-UTR, multiple putative poly-adenylation signals are predicted (underlined lower case letter). The deduced amino acid sequence of the ORF is shown below the nucleotide sequence for each codon. Two potential N-linked glycosylation sites on the N-terminus of the protein (Asp^{19} and Asp^{22}) are indicated by a ★; the ERY/DRY motif comprises the boxed letters; two potential Ser/Thr phosphorylation sites in the C-terminus of the protein (Ser^{334} and Ser^{344}) are labeled with ●.

24 h, and then treated with PK/PBAN peptides — including $PBAN_{1-33}NH_2$, $PBAN_{1-33}COOH$, $PBAN_{28-33}NH_2$ or Pss-PT — diluted in medium for 5 and/or 15 min as indicated on the figures. Signal transduction inhibitors to MEK1/2, PKC and Gαi were used, in some experiments, shortly before the peptide stimulation. The peptide stimulation was followed by protein extraction with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 100 mM NaF, 100 mM sodium orthovanadate, and 1 mM EGTA, pH 7.7). Protein concentration was determined with the DC protein assay kit (Bio-Rad) and equal amounts of protein extracts were resolved on 10% polyacrylamide-glycerol gels and electrophoresed at 50 V

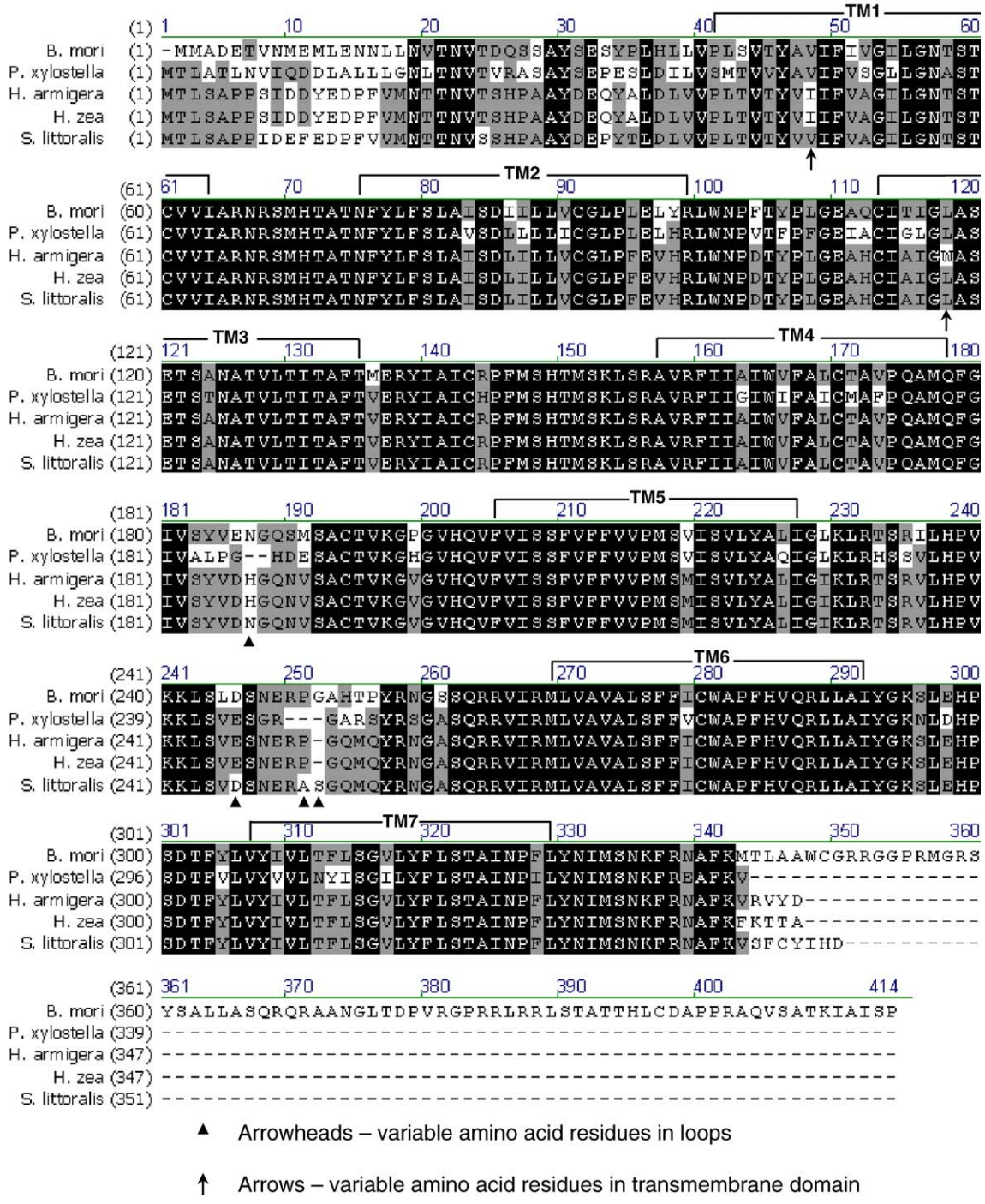

Fig. 2. Alignment of amino acid sequences of *S. littoralis*, *B. mori*, *P. xylostella*, *H. armigera* and *H. zea* PBAN-Rs. Identical amino acids are highlighted in dark gray and conserved amino acids in light gray for three or more sequences. The putative TM domains (TM1–TM7) for these PBAN-Rs were predicted by software analysis (TMHMM Server v.2.0,www.cbs.dtu.dk/services/TMHMM/) and are indicated by brackets above the amino acid sequences. Dashed lines indicate alignment gaps. Arrowheads point to amino acid sequences that are different in the loops whereas arrows point to amino acid residues that are different in the TM domains. GenBank accession numbers: *H. armigera*AY792036; *H. zea*AB181298; *B. mori*AY319852, *P. xylostella*AAY34744, *S. littoralis*DQ407742.

for 4 h. After transfer, the membranes were blocked with 10% skim milk and then probed overnight with primary antibodies specific to the phosphorylated ERKs (Cell Signaling, 1:2000 dilution) followed by goat anti-mouse or goat anti-rabbit polyclonal Abs conjugated to HRP at 1:10,000 dilution (Amersham Biosciences). Membranes were stripped and re-probed with Abs specific for the total ERK2 at a dilution of 1:4000 (Upstate Biotechnology) to ensure equal loading. The immunoblots were visualized by ECL and band intensities were determined by densitometric measurement.

3. Results

3.1. Cloning of S. littoralis PBAN-R cDNA

In order to isolate the full-length cDNA of the *S. littoralis* PBAN-R gene, degenerated PCR primers were designed, based on the high-homology regions of *H. zea* and *B. mori* PBAN-R genes. Total RNA was isolated from the 5th instar larvae of *S. littoralis*, transcribed into cDNA and used as a PCR template with these primers. The initial cloned fragment of ~400 bp was found to have 80–90% identity with the deduced amino acid sequence of known PBAN-Rs. We then designed gene-specific nested primers, based on the available sequence to amplify the 5′ and 3′ sequence by RACE. The 5′-RACE yielded a fragment containing the entire 5′-UTR and the beginning part of the ORF. The 3′-RACE yielded only one product of 700 bp which contained the tail of the ORF and the 3′-UTR region. To obtain the full-length ORF, additional primers were designed based on the sequence of the 5′- and 3′-UTR region. This amplification yielded an 1120 bp clone that, when assembled with the 5′-and 3′-UTR sequence, constituted the full cDNA sequence of ~1.55 kb (Fig. 1), containing a putative ORF of 1050 nucleotides encoding a 350-amino acid protein, flanked by a 5′-UTR of 188 bp and a 3′-UTR of 300 bp. The very first ATG present in the entire cDNA also encodes the initial codon of the putative ORF. Further analysis of the *S. littoralis* PBAN-R showed that this protein contains several characteristic features of type 1 seven TM GPCR (Fig. 2), including two potential N-linked glycosylation sites (Asp^{19} and Asp^{22}) that regulate the cell-surface localization and function (Vankoppen and Nathanson, 1990), and an ERY motif (aa 137–139) immediately following the third TM domain, which is a variant of the DRY motif that is responsible for G-protein interaction (Rosenkilde et al., 2005). We also identified two serine residues (Ser^{334} and Ser^{344}) on the C-terminal tail which are potentially responsible for the phosphorylation and internalization of the receptor upon ligand binding (Koenig and Edwardson, 1997).

In order to determine the hydropathic secondary structures of this protein, the amino sequence was processed by a TM domain prediction program TMHMM Ver. 2.0 (www.cbs.dtu.dk/services/TMHMM). The results show the presence of seven TM domains (Fig. 2), an extracellular N-terminus and an intracellular C-terminus which are characteristics of GPCRs. A BLAST homology search with the deduced amino acid sequence yielded several known insect GPCRs including the *H. zea* (92.3% identity), *B. mori* (81.7% identity) and *H. armigera* (92.6% identity) PBAN-Rs. Based on the above homologies, we designated this gene *S. littoralis* PBAN-R. However, when we compare the amino acid sequence of *S. littoralis* PBAN-R with those of *H. zea* and *H. armigera*, we can see that there are major differences in the N-terminal and C-terminal regions (Fig. 2). There are also several amino acid residue differences in the loops (Asn^{187}, Asp^{246}, Ala^{251}, Ser^{252}, arrowheads). In contrast, there are overall only two residue differences (Val^{48}, Leu^{118}, arrows) within all the TM domains.

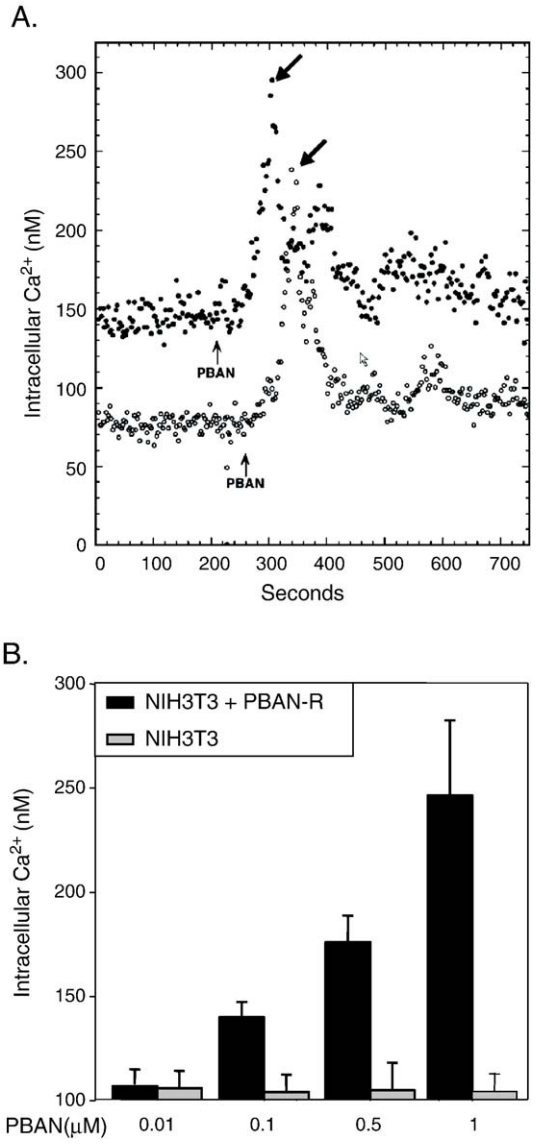

Fig. 3. PBAN stimulates intracellular Ca^{2+} level in PBAN-R-expressing NIH3T3 cells. NIH3T3 cells stably expressing the *S. littoralis* PBAN-R were loaded with the ratiometric Ca^{2+} indicator Fura-2, and then challenged with synthetic PBAN. Intracellular Ca^{2+} levels were monitored in real time (A) and the Ca^{2+} response was calibrated according to the maximum (ionophore) and minimum (EGTA) ratio obtained from the same cells following the peak Ca^{2+} response (A, arrows) by PBAN. Representative responses of two individual cells are illustrated here with curves composed of solid or empty circles, respectively. Ligand concentration-dependent peak Ca^{2+} response in transfected or wild-type NIH3T3 cells is determined (B), and the result is based on statistical validation of three independent experiments, each with $n = 12–20$.

3.2. Functional characterization of S. littoralis PBAN-R

In order to determine the functionality of this receptor, we used mutations to create a KOZAK sequence to this gene's ORF and cloned it into a retroviral expression vector pIB-2. The KOZAK sequence was added to enhance the expression of the PBAN-R gene in the mammalian cells we intend to use, including the murine NIH3T3 cells and human HEK293 cells. The retroviruses were used to infect the murine NIH3T3 cells that do not express PBAN-Rs. Infected cells were subjected to blasticidin selection for 7–10 days, and the cells that survived were expanded and used for the various functional assays.

It is known that PBAN signal transduction in pheromone gland cells involves an elevation of intracellular Ca^{2+} as second messenger (Rafaeli and Jurenka, 2003). Therefore, we examined whether PBAN could induce a Ca^{2+} response in the *S. littoralis* larva PBAN-R expressing NIH3T3 cells. PBAN-R-expressing or wild-type NIH3T3 cells were loaded with the ratiometric Ca^{2+} indicator Fura-2 and then challenged with various concentrations of synthetic full-length PBAN. Changes in single-cell intracellular free Ca^{2+} levels were monitored in real time with a fluorescent microscope. PBAN induced a rapid Ca^{2+} response in NIH3T3 cells expressing *S. littoralis* PBAN-R (Fig. 3A) and the Ca^{2+} response was found to be ligand concentration-dependent (Fig. 3B). We also challenged the wild-type NIH3T3 cells that did not express the *S. littoralis* PBAN-R with PBAN up to 1 μM, but did not observe a Ca^{2+} response under any of the tested conditions (Fig. 3B). The results suggest that the Ca^{2+} increase is due to extracellular Ca^{2+} influx rather than from intracellular sources because if the assay buffer, which normally contains 1.2 mM free Ca^{2+}, is stripped of Ca^{2+} we do not observe a significant response to PBAN ligand stimulation.

GPCRs often trigger signal transduction mechanisms that phosphorylate/activate MAP kinases. To determine further whether PBAN can stimulate PBAN-R to phosphorylate MAP kinases we used the NIH3T3 cells that express the *S. littoralis* PBAN-R constitutively and treated them with PBAN for several different time intervals. The phosphorylation of ERK 1/2 (MAP kinases) was determined by immunoblot analysis using an antibody specific to the phosphorylated forms of the ERKs. With a PBAN concentration of 200 nM, which stimulates Ca^{2+} response in these PBAN-R expressing cells, the ERK 1/2 reached peak activation between 5 and 10 min after the PBAN stimulation and then declined to basal level rapidly thereafter (Fig. 4A). Similar experiments were performed on NIH3T3 cells that did not express the *S. littoralis* PBAN-R and no MAP kinase activation was observed. It had previously been shown that numerous chemokines activate MAP kinase in NIH3T3 cells through coupling of their GPCRs to a signal transduction pathway that involves Gαi, and protein kinase C (PKC), and that leads to ERK 1/2 phosphorylation (Li et al., 2004). To test this possibility, we used signal transduction inhibitors to these various molecules. Gö6983, a potent and specific PKC inhibitor, significantly inhibited PBAN-induced MAP kinase activation. PD98059, an ERK upstream activator MEK1/2 inhibitor with an IC_{50} of approximately 2 μM *in vitro* (Calbiochem), was used here at 3 μM as a partial inhibitor for PBAN activation of ERK (Fig. 4B). Higher concentration of PD98059 was not used to avoid compromising cell vitality in these experiments. To our surprise on the other hand, a Gαi inhibitor, pertussis toxin, did not inhibit the activation of the receptor unlike numerous chemokine GPCR-induced signal transduction pathway (data not shown). These results collectively indicate that agonist stimulation of PBAN-R can mobilize Ca^{2+} as a secondary messenger in a Gαi-independent way, which is then coupled to PKC activation and PKC-dependent MAP kinase activation.

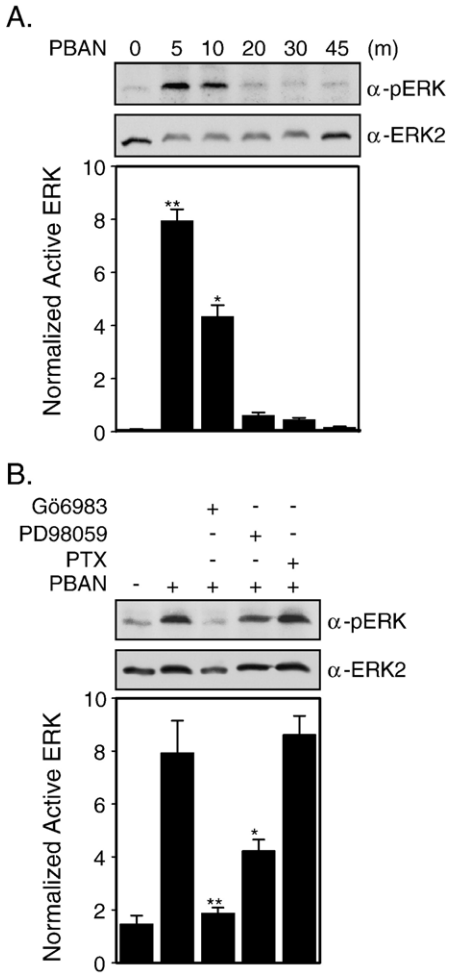

Fig. 4. PBAN activates MAP kinase signaling in mammalian cells expressing PBAN-R. NIH3T3 cells stably expressing *S. littoralis* PBAN-R were exposed to PBAN (200 nM) and ERK 1/2 activation was determined by immunoblot analysis with an antibody specific for the phosphor-ERKs. (A) Cells were treated with PBAN for the indicated time intervals and the ERK 1/2 phosphorylation was determined by immunoblot (upper panel) with densitometric measurement (lower panel). (B) Cells were treated with PBAN (200 nM) in the presence of various pathway inhibitors to further confirm that PBAN-R stimulates ERK 1/2 activation. Inhibitors were applied 30 min before PBAN treatment, the samples were collected 5 min after PBAN treatment, and ERK 1/2 activation was detected by immunoblot (upper panel) with densitometric measurement (lower panel). The inhibitors were used at the following concentrations: PD98059 (3 μM), a specific inhibitor of MEK 1/2 of ERK signaling pathway; pertussis toxin (100 nM), an inhibitor of Gαi; Gö6983 (0.3 μM), a selective and potent inhibitor of PKC.

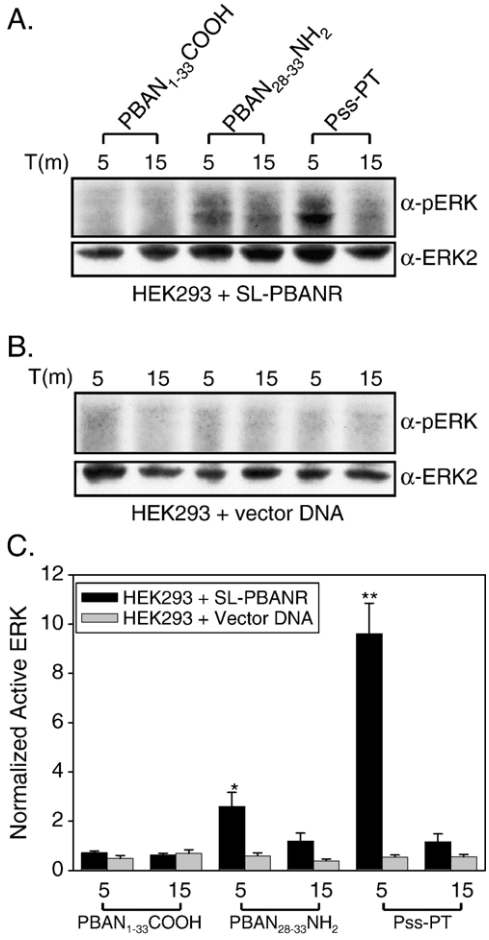

Fig. 5. PBAN-R responds to multiple neuropeptide agonists. HEK293 cells transfected with PBAN-R (A) or non-transfected (B) were challenged with several PBAN-derived or -related peptides: $PBAN_{1-33}COOH$ (an inactive form of PBAN), $PBAN_{28-33}NH_2$ and a PBAN-R agonist Pss-PT (pheromonotropin), which is a member of the PK/PBAN family, at 1 μM for the indicated times. ERK activation by these peptides was determined by immunoblot analysis and then measured by densitometry (C).

To further determine the specificity of this receptor, HEK293 cells expressing SL-PBAN-R were challenged with several peptides, including: an inactive form of PBAN (free-acid $PBAN_{1-33}COOH$) (Altstein et al., 1995, 1996), a PBAN-derived C-terminal 6-amino acid peptide ($PBAN_{28-33}NH_2$) that is a weaker agonist of PBAN-R than the full-length PBAN (Altstein et al., 1995, 1996), and a Pss-PT that is a strong agonist of PBAN-R in *S. littoralis* (Altstein et al., in press). These HEK293 cells can be transfected at a high efficiency of up to 95% and we found that these receptor-expressing cells can respond to PBAN stimulation in a similar way to NIH3T3 cells. However when these cells are treated with different peptides, Pss-PT stimulated a robust ERK activation 5 min after addition of the ligand. $PBAN_{28-33}NH_2$ stimulated a moderate activation of ERK whereas the inactive form $PBAN_{1-33}COOH$, failed to induce ERK activation (Fig. 5A). In untransfected cells, none of these peptides were able to stimulate significant ERK phosphorylation (Fig. 5B).

In summary, we have cloned the first PBAN-R gene from insect larval tissue, and showed that this receptor can be activated by the PK/PBAN neuropeptide family. Our PBAN-R resembles the native pheromonotropic and melanotropic receptor with respect to its response to different PK/PBAN peptides, and can stimulate Ca^{2+} mobilization and downstream signaling, such as ERK activation.

4. Discussion

The PK/PBAN family is a multi-functional family of peptides that plays a major role in the physiology of insects. Members of the family have been shown to stimulate cuticular melanization in moth larvae, to induce sex pheromone biosynthesis in adult female moths, to mediate key functions associated with feeding (gut muscle contractions) and control of development (pupariation and diapause) in a variety of insects (moths, cockroaches, locusts and flies). For reviews see Altstein (2004) and Rafaeli and Jurenka (2003). Despite the considerable amount of knowledge available about the expression, localization, function and signal transduction of the PK/PBAN peptides in these insects, characterization of their receptor(s) had been elusive until the recent cloning of PBAN-R from the pheromone gland of adult female moths of the species *H. zea*, *B. mori*, *H. armigera* and *P. xylostella*. In the present study we isolated and characterized another PBAN-R gene from *S. littoralis* larvae, by using PCR and RACE. This 1.55 kb transcript contains an ORF encoding a protein of 350 amino acids and when expressed in mammalian cells, it can mediate PBAN-induced Ca^{2+} response in a dose-dependent manner. Moreover, PBAN also stimulates PKC-dependent MAP kinase activation in response to various members of the PK/PBAN family.

The *S. littoralis* larval PBAN-R shared significant amino acid identity to other PBAN-Rs isolated from the pheromone gland of adult female moths (92, 81, 92 and 72% amino acid identity to *H. zea*, *B. mori, H. armigera and P. xylostella* PBAN-R, respectively). The major variations were in the extracellular N-terminus and the C-terminal tail. Sporadic differences were also present on the extracellular or intracellular loops, whereas the predicted TM domains differed in only two amino acid residues from those of *H. zea* and *H. armigera* (as indicated with arrows/arrowheads in Fig. 2). The structural mechanisms of the ligand/GPCR interaction are still not clear, because of the lack of knowledge of the high resolution structures of these seven TM receptors. However, an accumulation of data suggests that type-1 seven TM GPCRs can be divided into three functional domains, namely, extracellular N-terminus/loops, TM helices, and the intracellular loops/C-terminal tail, all of which can be activated in a two-state docking/activation process. The N-terminal regions and extracellular loops of GPCRs are responsible for ligand docking/interaction (Leff, 1995). The structural reconfiguration of the TM domain upon ligand binding is thought to be responsible for the TM relay of a signal, and the intracellular loops and tails are responsible for G-protein interaction and receptor desensitization and internalization. The PK/PBAN family of neuropeptides is diverse both in function and sequence, but a common motif, FXPRLamide, on the C-terminus is critical for its functioning (Altstein, 2004; Rafaeli and Jurenka, 2003). It has been shown

that the FXPRLamide motif is the essential minimal part of the ligand that can activate their receptors (Altstein et al., 1995, 1996), therefore, the remaining amino acids of the ligand might play different roles such as facilitating the docking of the ligand to the receptor. The major difference observed on the N-terminal region of the PBAN-R may account for its optimal conspecific ligand/receptor docking and interaction. The highly conserved TM helices between different PBAN-Rs might form a precise ligand interaction pocket that is required for the FXPRLamide motif-induced receptor conformational change and receptor activation. The intracellular loops form a highly conserved interface for efficient G-protein interaction, but the C-terminal tail is somewhat diverse. In most peptides or small molecule GPCRs, after a ligand activates the receptor, the C-terminus is phosphorylated on serine and threonine residues and targeted into clathrin-coated membrane pits for internalization. It has also been shown that, the extra-long C-terminal tail of *B. mori* PBAN-R functions in regulating the internalization process upon ligand activation (Hull et al., 2005).

The differences in the N-terminal region and extracellular loops of the *S. littoralis* larval PBAN-R and the pheromone gland receptors account, most likely, for the differing bioactivity patterns of the PK/PBAN family members. For example, comparison of the *in vivo* pheromonotropic and melanotropic activities of PBAN, Pss-PT, LPK and Lom-MT-II in a variety of moth species revealed that some peptides, e.g., Lom-MT-II, were highly potent in stimulating sex pheromone biosynthesis and much less so in inducing cuticular melanization (Altstein et al., in press). Similar results were obtained with a variety of PBAN-derived analogs that exhibited selective agonistic and/or antagonistic activity, i.e., a capability to inhibit or stimulate one function and not the other (Altstein et al., in press). The hypothesis was further supported by the behavior of a library of backbone cyclic (BBC) conformationally constrained PBAN-derived peptides, which, because of their rigid structure are much more receptor selective. The BBC peptides, all of which have the same primary sequence, same bridge location, and chemistry but differ from each other in their bridge sizes (Altstein et al., 1999), exhibited selective agonistic and antagonistic pheromonotropic and melanotropic activities (Ben-Aziz et al., 2006). It should be noted, however, that although the above results do imply that the differing sequences in the N-terminal and extracellular region may affect the docking and interaction of the peptide ligands with the receptors, which in turn may account for the differing and selective activities of the tested peptides, the possibility that the different assay conditions accounted for the differences in bioactivity should not be excluded.

The functional assays of this receptor were done by stably expressing this protein in NIH3T3 and transiently in HEK293 cells. Synthetic PBAN induces a robust intracellular Ca^{2+} response as determined by real-time imaging, similar to what has been found in the insect pheromone gland cells, where Ca^{2+} influx is an important secondary messenger for the PBAN signaling of sex pheromone biosynthesis. The PBAN-induced MAP kinase activation can be abrogated by a potent and specific PKC inhibitor, thus indicating the efficient coupling of Ca^{2+} elevation, PKC activation and subsequent MAP kinase activation within the PBAN-induced signal transduction. Currently, our knowledge of the signal transduction events that occur after PBAN binds to its receptor has been based on studies of the pheromone gland tissue in association with sex pheromone biosynthesis. The present study is the first to address the signal transduction mechanism of the PBAN-R in *S. littoralis* larvae. However, although the involvement of Ca^{2+} flux in the signal transduction process has been demonstrated, the question remains of whether the process is mediated via cAMP (as in the case of Heliothinae species) or whether the Ca^{2+} directly activates downstream events independently of cAMP activation.

The above assays have also demonstrated that PBAN and other peptides of the PK/PBAN family can trigger MAP kinase activation in a PKC-dependent manner. Thus, it is interesting to note the high correlation between the responses of the cloned receptor and those of the native (*in vivo*) receptor to various PK/PBAN peptides and analogs. Both receptors exhibited a strong response to the full-length PBAN and Pss-PT (Altstein et al., in press), a lower response in the presence of the C-terminal-derived $PBAN_{28-33}NH_2$ (Altstein et al., 1996), and no response in the presence of the C-terminal free-acid analog (Altstein et al., 1996). The similar activity patterns of the different peptides, both *in vitro* and *in vivo*, provide another proof of the functional resemblance between the cloned and the native PBAN-Rs.

Previous studies focused exclusively on the expression and characterization of PBAN-R obtained from pheromone gland cells of adult female moths of various species (Choi et al., 2003; Hull et al., 2004) which mediates PK/PBAN-stimulated sex pheromone production. Here we report, for the first time, the expression and characterization of another PBAN-R, which is expressed in the larval stage and which most likely, is the receptor involved in other PK/PBAN functions during the insect larval stage, such as cuticular melanization (Ben-Aziz et al., 2005) or potential regulation of pupal diapause (Sun et al., 2005; Xu and Denlinger, 2003). Diapause is an adaptive stage in which the insects temporarily stagnate, in order to survive an unfavorable environmental condition. Recent studies have found that PK/PBAN peptides play a role in pupal diapause, which is reflected by changes in the expression levels of PK/PBAN-related peptides (low in diapause destined insects and high in non-diapause destined insects) at different larval development stages (Sun et al., 2005; Xu and Denlinger, 2003). Although the exact diapause- and melanization-inducing pathways are yet to be identified, the expression of the PBAN-R in the larval stage suggests its functional role in these processes.

In summary, a functional receptor for the PK/PBAN peptides has been cloned from the *S. littoralis* larvae. This receptor, when activated by PK/PBAN peptides, stimulates signal transduction pathways that are characteristic of the PBAN family of receptors. Furthermore, our results provided supporting evidence that PBAN-like neuropeptides can potentially regulate numerous physiological events, such as melanization and diapause, during the Lepidopteran larval stages, by demonstrating the presence of a functional receptor that can be activated by PBAN. The receptor that was cloned in the

present study, together with those that had already been cloned, provide a basis for gaining further information on structural homologies/heterologies among receptor sub-types and sub-families of the PK/PBAN peptides and facilitate our understanding of the cell-specific responses and functional diversities of this diverse Np family.

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