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Identification of a new member of the PBAN family of neuropeptides from the fire ant, *Solenopsis invicta*

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Abstract

Neuropeptide hormones produced by neurosecretory cells in the central or peripheral nervous systems regulate various physiological and behavioral events during insect development and reproduction. PBAN/Pyrokinin is a major neuropeptide family, characterized by a 5-amino-acid C-terminal sequence, FXPRLamide. This family of peptides has been implicated in regulating various physiological functions including, pheromone biosynthesis, muscle contraction, diapause induction or termination, melanization, and puparium formation in different insect species. In the present study, we report a new member of the PBAN family from the red imported fire ant, *Solenopsis invicta*, Soi-PBAN, composed of 26-AA (GSGEDLSYGDAYEVDEDDHPLFVPRL). Three additional peptides were deduced from Soi-PBAN cDNA: 15-AA (TSQDIASGMWFGPRL), 8-AA (QPQFTPRL) and 9-AA (LPWIPSPRL), that correspond to diapause hormone (DH), β-neuropeptide (NP), and γ-NP, which are found in many lepidopteran moths. Five peptides, DH, α, β, γ NPs, and PBAN are encoded from PBAN genes of lepidopteran moths, but in the fire ant the α-NP is missing. Each of the four synthetic peptides from the fire ant Soi-PBAN cDNA showed significant pheromonotropic activity in a moth model, indicating that these peptides are cross-reactive. Soi-β-NP induced the highest amount of pheromone production of the four peptides evaluated. The Soi-DH homologue had the lowest pheromonotropic activity, but was still significantly greater than control values. When the deduced amino acid sequences (entire ORF domains) from Soi-PBAN cDNA were compared with other known sequences, the fire ant was most similar to the honey bee, but phylogenetically distant from moth and beetle species. Soi-PBAN (26-AA) unlike the other three peptides shows a low degree of sequence identity with honeybee PBAN (33-AA). Based on the amino acid sequences encoded from insect PBAN genes identified to date, neuropeptide diversity is correlated with the taxonomic or phylogenetic classification of Insecta. From the present study we report the first neuropeptide identified and characterized from the central nervous system of Formicidae.

Keywords: Fire ant, PBAN, Neuropeptide, Pheromone, *Solenopsis invicta*.

Introduction

Neuropeptides are the largest group of insect hormones and are produced in the central and peripheral nervous systems, where they are released into the hemolymph, affecting development and reproduction. A variety of peptide families have been identified from insects (Gade *et al*., 1997). One of these families is the Pheromone Biosynthesis Activating Neuropeptide (PBAN)/Pyrokinin family defined by a conserved C-terminal pentapeptide (FXPRLamide) that is the active core fragment for peptide function (Raina & Kempe, 1992). A pyrokinin (leucopyrokinin) from the cockroach, *Leucophaea maderae*, was first isolated and characterized (Holman *et al*., 1986) as a myotropin with subsequent myotropic peptides being identified from various insect orders (Nachman *et al*., 1986). PBAN has been the subject of a great deal of interest especially for lepidopteran moths, since the first PBAN was identified from *Helicoverpa zea* adults two decades ago (Raina *et al*., 1989). Now, the PBAN/pyrokinin peptide family with a FXPRLamide functional epitope is expected to be widely distributed in Insecta with various physiological functions already documented: (1) stimulation of pheromone biosynthesis in female moths (Raina *et al*., 1989); (2) induction of melanization in moth larvae (Matsumoto *et al*., 1990;

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The cDNA sequence reported in this paper has been deposited in the GenBank database (Accession no. FJ223176)

Raina *et al*., 2003); (3) induction of embryonic diapause in *Bombyx mori* (Suwan *et al*., 1994); (4) stimulation of visceral muscle contraction in cockroaches (Predel & Nachman, 2001); (5) acceleration of puparium formation in the flesh fly (Zdarek *et al*., 1997); and (6) termination of development of pupal diapause in heliothine moths (Xu & Denlinger, 2003). The PBAN/pyrokinin family peptides are cross-reactive in that each peptide can activate all physiological functions noted above in experimental models.

The neurohormonal action of PBAN for pheromone biosynthesis in lepidopteran moths is well studied (Rafaeli & Jurenka, 2003). PBAN is synthesized in the subesophageal ganglion (SG) and is released into the hemolymph via the corpora cardiaca (CC), a neurohemal organ in *H. zea* (Raina and Klun, 1984). The first PBAN identified was the 33-amino acid peptide from *H. zea*, Hez-PBAN (*Helicoverpa zea* PBAN) (Raina *et al*., 1989). Subsequently PBAN amino acid sequences were determined from *B. mori* (Kitamura *et al*., 1989; Kitamura *et al*., 1990) and *Lymantria dispar* (Masler *et al*., 1994), through direct isolation and purification of peptides. Using DNA cloning tools more PBAN encoding genes have been identified from the moths, *B. mori* (Kawano *et al*., 1992; Sato *et al*., 1993), *H. zea* (Davis *et al*., 1992; Ma *et al*., 1994), *Mamestra brassicae* (Jacquin-Joly *et al*., 1998), *Helicoverpa assulta* (Choi *et al*., 1998), *Helicoverpa armigera* (Choi, 1999; Zhang *et al*., 2004), *Agrotis ipsilon* (Duportets *et al*., 1999), *Bombyx mandarina* (Xu *et al*., 1999), *Spodoptera littoralis* (Iglesias *et al*., 2002), *Heliothis virescens* (Xu & Denlinger, 2003), *Manduca sexta* (Xu & Denlinger, 2004), *Adoxophyes* sp. (Choi *et al*., 2004), *Samia cynthia ricini* (Wei *et al*., 2004), *Plutella xylostella* (Lee & Boo, 2005), *Ascotis selenaria cretacea* (Kawai *et al*., 2007), *Clostera anastomosis* (Jing *et al*., 2007), *Spodoptera exigua* (Xu *et al*., 2007), *Orgyia thyellina* (Uehara *et al*., 2007) and *Antheraea pernyi* (Wei *et al*., 2008). From the identification of PBAN cDNAs, PBAN, diapause hormone (DH) and three additional FXPR/KL neuropeptides (NPs: α, β, γ) were deduced from the same gene and are well conserved in moths. Besides lepidopteran moths, no other insect group has been demonstrated to use PBAN for pheromone production or regulation. Recently, two non-lepidopteran PBAN/Pyrokinin peptides have been identified from genome research in the red flour beetle, *Tribolium casteneum* (Li *et al*., 2008) and the honeybee, *Apis mellifera* (Hummon *et al*., 2006); however, no physiological function has been determined for these peptides.

Social insects utilize sophisticated pheromonal communication to maintain colony cohesiveness and sociality. All ants are social and the red imported fire ant, *So. invicta*, is an economically important invasive pest ant species in the United States and elsewhere around the world, e.g. Australia and China. This opportunistic omnivore occurs in very large numbers in its invasive range and prefers disturbed habitats (Vander Meer *et al*., 2007). The affected economic sectors include: residential households, electric and communication systems, agriculture, golf courses, and recreational areas. The fire ant is probably the most studied ant species in the world and a great deal is known about the pheromone systems used to reduce reproductive competition, recruit to resources, and other pheromones necessary to maintain colony social structure and territoriality (Vander Meer & Alonso, 1998, 2002; Vargo, 1998). In spite of decades of study on fire ant pheromones, virtually nothing is known about how pheromone production and release are regulated, nor whether protein hormones, especially neuropeptides, are involved in key physiological and endocrinal processes during development.

We previously demonstrated the presence of PBAN/pyrokinin family peptides and localization of immunoreactive neurons by applying an immunocytochemical technique to the central nervous system of the fire ant (Choi *et al*., 2009). In the present study, we report a new member of the PBAN/pyrokinin family from the red imported fire ant, *So. invicta*, PBAN and three additional F/PXPRL type peptides from the Soi-PBAN gene. We demonstrate that synthetic peptides deduced from Soi-PBAN cDNA stimulate significant pheromone production in a moth model. This is the first time that a PBAN/pyrokinin family peptide from an ant species has been shown to have pheromonotropic activity.

Results

Structure of PBAN cDNA

A PCR amplified 360-bp long product was obtained that included a possible PBAN and three F/PXPRL peptide domains. Based on this sequence additional gene specific primers were designed to extend the 5′ and 3′ ends of the PBAN cDNA. Using 5′- & 3′- RACE, a 754bp-long full cDNA was obtained that contained an entire open reading frame (ORF) of 531 nucleotides encoding 176 amino acids from the first initiation codon (ATG) to the termination codon (TAG) indicated in the boxes (Fig. 1). The TATA box was located 34-bp upstream of the transcription start site in the cDNA. The cleavage site for the signal peptide is predicted between the first 29 and 30 amino acids. The cDNA has four putative peptides based on six possible endoproteolytic cleavage sites (Veenstra, 2000; Southey *et al*., 2008) K^{62}-R^{63}, G^{79}-K^{80}-R^{81}, K^{116}-R^{117}, G^{126}-R^{127}, G^{154}-R^{155}-R^{156}, and G^{166}-R^{167}, indicated in italics in Fig. 1. The peptides cleaved are predicted to have a C-terminal amide group provided by glycine (G).

The third domain with a 26-amino acid (AA), GSGEDLSYGDAYEVDEDDHPLFVPRLamide, is considered a putative PBAN homologue, *So. invicta* PBAN (Soi-PBAN). There are three additional putative peptides deduced from the Soi-PBAN cDNA. One of these peptides, 15-AA, TSQDIASGMWFGPRLamide, is homologous to the diapause

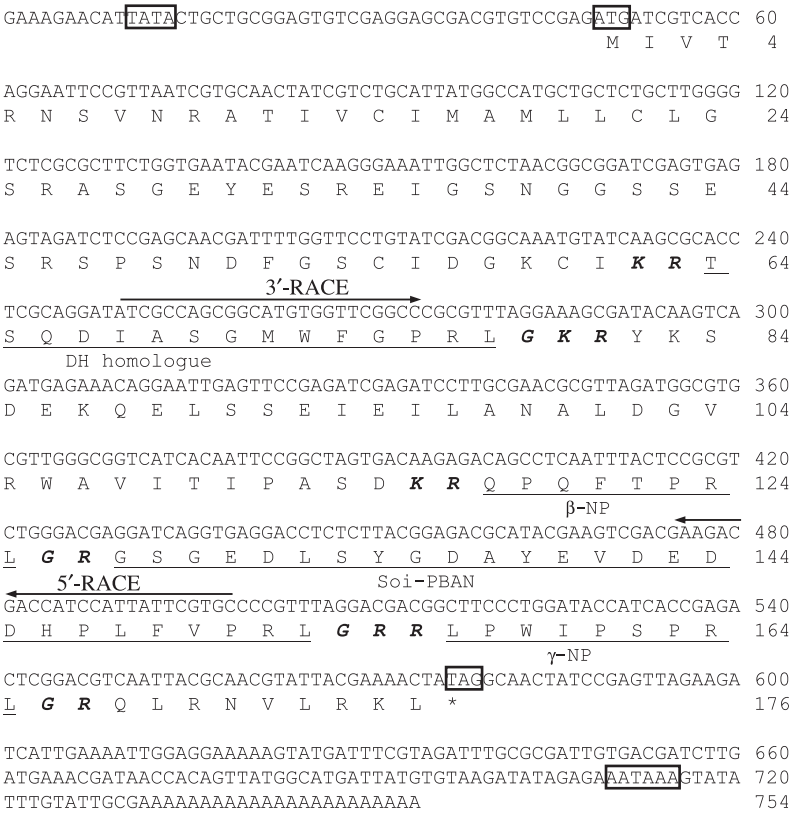

Figure 1. Soi-PBAN cDNA and deduced 176 amino acid sequence possessing putative Soi- DH, PBAN, β and γ neuropeptides (underlined). Six predicted endoproteolytic cleavage sites, and the priming sites for 5′- & 3′-RACEs are indicated by bold italic and arrows, respectively. The first 29 and 30 amino acids are predicted to cleave for signal peptide. The first ATG was used as the initiation codon, TATA box as promoter binding site, TAG as the termination codon, and AATAAA as the polyadenylation signal (indicated in boxes).

hormone (DH) or PBAN-encoding gene neuropeptide-24 (PGN-24) domain in lepidopteran moths (Fig. 2). So far, this type of peptide possesses a conserved WFGPRL sequence in the C-terminus, except in *Adoxophyes* moth species (Fig. 2). The 8-AA, QPQFTPRL positioned in the second domain, has a relatively short sequence and is likely a β type neuropeptide (β-NP) with an FTPRLamide at the C-terminus. The fourth domain, 9-AA, LPWIPSPRLamide, has a Proline (P) instead of Phenylalanine (F) at the C-terminus of the pentapeptide, and likely corresponds to the γ-NP homologue in moths (Figs 1 and 2). Unlike moth PBAN genes the fire ant PBAN cDNA does not contain a α-NP homologue, VIFTPKLamide, which is highly conserved in moths (Figs 1 and 2). When the fire ant is compared to the honeybee (both species belong to the Order Hymenoptera) the length of the *So. invicta* PBAN gene (176-AA) is shorter than *A. mellifera* (195-AA). The latter is similar in size to ORFs found in moth PBAN genes (Fig. 3). The entire ORF sequence identity from the two species showed 56% similarity.

Pheromonotropic activity by So. invicta neuropeptides in moths

Generally, the *H. zea* female does not produce pheromones during the photophase or when decapitated. However, the amount of pheromone produced after injection with 3 pmol of Soi-PBAN or the three other peptides encoded from Soi-PBAN cDNA were all significantly higher than the saline injections ($P \leq 0.0154$), but lower than for 3 pmol synthetic Hez-PBAN (*H. zea* PBAN) injections (Fig. 4). The pheromonotropic activities of Soi-PBAN, β- and γ- neuropeptides (NP) were not significantly different. Soi-β-NP, which is the shortest peptide (8-AA), elicited the greatest pheromonotropic activity in the moths (Fig. 4). Soi-DH corresponded to a homologue of the diapause hormone (DH) or PGN-24 in moths and had the lowest pheromonotropic activity, but still significantly greater than saline injections. These results indicated that all putative fire ant PBAN/pyrokinin neuropeptides can stimulate pheromone biosynthesis in moths.

Discussion

Although PBAN and pyrokinin family peptides have been found independently from many insects based on their different functions, they are both characterized by a conserved pentapeptide (FXPRLamide) in their C-termini. These peptides are expected to be ubiquitous in insects and affect various physiological events, such as pheromone production and diapause during development and reproduction. However, regulation of pheromone biosynthesis by PBAN has only been determined in lepidopteran moths (Rafaeli & Jurenka, 2003). Although two new PBAN/pyrokinin-like peptides have been recently found from Hymenoptera

Insect PBANs and Neuropeptides

	DH	α-NP	β-NP	PBAN	γ-NP
H. zea	NDVKDGAASG-AHSDRLG-LWFGPRL	VIFTPKL	SLAYDD---KSF-ENVEFTPRL	LSDDMPATPADQEMYRQD--PEQIDSRT-KYFSPRL	--TMN-FSPRL
H. assulta	NDVKDGAASG-AHSDRLG-LWFGPRL	VIFTPKL	SLAYDD---KSF-ENVEFTPRL	LSDDMPATPADQEMYRQD--PEQIDSRT-KYFSPRL	--TMN-FSPRL
H. armigera	NDVKDGGAASGAHSDRLG-LWFGPRL	VIFTPKL	SLAYDD---KSF-ENVEFTPRL	LSDDMPATPADQEMYRQD--PEQIDSRT-KYFSPRL	--TMN-FSPRL
H. virescens	NDDKDGAASG-AHSDRLG-LWFGPRL	VIFTPKL	SLSYDD---KSF-ENVEFTPRL	LADDMPATPADQEMYRQD--PEQIDSRRTKYFSPRL	--TMN-FSPRL
A. ipsilon	NDVKDGGADRGAHSDRGG-MWFGPRI	VIFTPKL	SLSYED---KMF-DNVEFTPRL	LADDTPATPADQEMYRPD--PEQIDSRT-KYFSPRL	--TMN-FSPRL
M. brassicae	----------	VIFTPKL	SLAYDD---KVF-ENVEFTPRL	LADDMPATPADQEMYRPD--PEQIDSRT-KYFSPRL	--TMN-FSPRL
S. littoralis	NEIKDGGSDRGAHSDRAG-LWFGPRL	VIFTPKL	SLAYDD---KVF-ENVEFTPRL	LADDMPATPADQELYRPD--PDQIDSRT-KYFSPRL	--TMN-FSPRL
S. exigua	NEIKDGGSDRGAHSDRAG-LWFGPRL	VIFTPKL	SLAYDD---KVF-ENVEFTPRL	LSDDMPATPADQELYRPD--PDQIDSRT-KYFSPRL	--TMN-FSPRL
C. anastomosis	NTMKDGGADRGAHSDRGG-LWFGPRL	VVFTPKL	SMAYDD---KSY-ENVEFTPRL	LADDMPATPSDQEYYRQD--PEQIDSRS-NYFSPRL	--TMT-LTPRL
O. thyellina	NDVKDDGQDRVAHSDRGGQLWFGPRL	VIFTPKL	SLSTYEE--KLY-DNVEFTPRL	LSDDMPATPPDQEYYRPD--PEQIDSRT-KYFSPRL	--TMT-FSPRL
A. pernyi	NDIKDEG-DKGAHSDRGS-LWFGPRL	VIFTPKL	SIGDIYQEKRTY-ENVEFTPRL	LSDDMPATPKDQEMYHQD--PEQVDTRT-RYFSPRL	--TIT-FSPRL
B. mori	TDMKDES-DRGAHSERGA-LWFGPRL	IIFTPKL	SVA--KP--QTH-ESLEFIPRL	LSEDMPATPADQEMYQPD--PEEMESRT-RYFSPRL	--TMS-FSPRL
B. Mandarina	TDMKDES-DRGAHSERGA-LWFGPRL	IIFTPKL	SVA--KP------ENFEFIPRL	LSEDMPATPADQEIYQPD--PEVMESRT-RYFSPRL	--TMS-FSPRL
M. sexta	NDIKDEG-DRGAHSDRGA-LWFGPRL	VIFTPEL	SLDDSTQEKRVFYENFEFTPRL	ISEDMPATPSDQEYPMYHPDPEQIDTRT-RYFSPRL	---TH-FSPRL
S. c. ricini	NDVKDEG-DRGAHSDRGS-LWFGPRL	VIFTPKL	--ASNAYQEKRTYENVEFTPRL	LTEDMPATPTDQE--MFDQDPEQIDTRT-RYFSPRL	--TMT-FSPRL
Adoxophyes. sp.	NFKEENF-DRNIRSGRAN-VVFKPIL	VIFTPKL	--SMEDPYEEKRSYDVDFTPRL	QSEAVTS--SD-EQVYRQDMSP-VDGRL-KYFSPRL	--TVKLT-PRL
P. xylostella	DDLKDEDIQRDAR-DRAS-MWFGPRL	VIFTPKL	-----NADEQQQS--VDFTPRL	RLKDSGLAPPD-E-YRT---PELLDARA-QYFSPRL	GGSM-TFSPRL
A. s. cretacea	NDLK-EDGEREANSDRQG-LWFGPRL	VIFTPKL	----------SVDFTPRL	QLVDDVPQRQQIEEDRL-------GSRT-RFFSPRL	-TTMN-FSPRL
L. dispar	----------	-------	----------	LADDMPATMADQEVYRPE--PEQIDSRNK-YFSPRL	----------
P. separata	----------	-------	----KLSYDDKVFENVEFTPRL	----------	----------
S. invicta	TSQDIAS----------GMWFGPRL	------	QP-Q----------FTPRL	GSGEDL---SYGDAY----EVDEDD---HPLFVPRL	--LPWIPSPRL
A. melifera	TSQDITS----------GMWFGPRL	------	QITQ----------FTPRL	ESGEDYF--SYGFPKD-QEELYTEEQIYLPLFASRL	--VPWTPSPRL
T. castaneum	TPHESSVPNERNDDSKETYFWFGPRL	------	HVVN----------FTPRL	----------SPPFAPRL	--HSSPFSPRL

Lepidoptera (top) and Hymenoptera

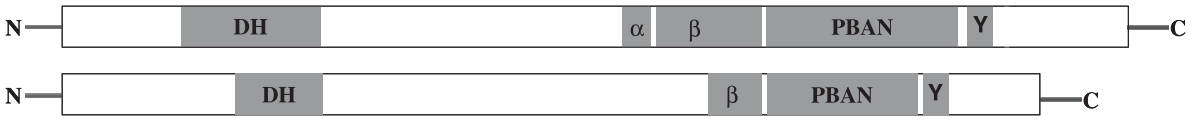

Figure 2. Comparison of PBAN and other neuropeptides in moths (top) and schematic diagram of PBAN-DH gene structures from Lepidoptera and Hymenoptera (bottom). The full PBAN genes of *Lymantria dispar* (Masler *et al.*, 1994) and Pss-PT of *Plutella separata* (Matsumoto *et al.*, 1992) have not been identified. Compared full or partial amino acid sequences are from lepidopteran species, *Helicoverpa zea* (Ma *et al.*, 1994), *Helicoverpa assulta* (Choi *et al.*, 1998), *Helicoverpa armigera* (Zhang *et al.*, 2004), *Heliothis virescens* (Xu & Denlinger, 2003), *Agrotis ipsilon* (Duportets *et al.*, 1999), *Mamestra brassicae* (Jacquin-Joly *et al.*, 1998), *Spodoptera littoralis* (Iglesias *et al.*, 2002), *Spodoptera exigua* (Xu *et al.*, 2007), *Closteria anastomosis* (Jing *et al.*, 2007), *Orgyia thyellina* (Uehara *et al.*, 2007), *Antheraea pernyi* (Wei *et al.*, 2008), *Bombyx mori* (Sato *et al.*, 1993), *Bombyx mandarina* (Xu *et al.*, 1999), *Manducta sexta* (Xu & Denlinger, 2004), *Samia cynthia ricini* (Wei *et al.*, 2004), Adoxophyes sp. that has not been specified (Choi *et al.*, 2004), *Plutella xylostella* (Lee & Boo, 2005), *Ascotis selenaria cretacea* (Kawai *et al.*, 2007), hymenopteran species, *Solenopsis invicta* (this study), *Apis mellifera* (Hummon *et al.*, 2006), and pyrokinin from coleopteran species, *Tribolium casteneum* (Li *et al.*, 2008). DH: Diapause Hormone, NP: Neuropeptide, PBAN: Pheromone Biosynthesis Activating Neuropeptide.

and Coleoptera (Hummon *et al.*, 2006; Li *et al.*, 2008), their physiological role(s) have not been characterized. During the last four decades the fire ant has been intensively studied, due to the broad range of economic damage caused by this destructive, omnivorous species. As in other social insects the fire ant has evolved a sophisticated pheromonal communication system to maintain colony cohesiveness and sociality. Several pheromone components from fire ants have been identified (Vander Meer & Alonso, 1998). However, knowledge of pheromone production and release is still unknown, as is the role of neuropeptides in the physiology of development. This study lays the foundation necessary to start the search for a physiological role for PBAN related to pheromone production or other endocrinal processes in the fire ant. Here we report the first identification and structural characterization of a PBAN/pyrokinin family peptide from Hymenoptera, specifically, *So. invicta* PBAN (Soi-PBAN).

To date, PBANs from 19 species of Lepidopteran moths have been identified: super families Noctuoidae (Noctuidae: eight species; Lymantridae: two species; Notodontidae: one species), Bombycoidae (Saturniidae: two species; Lymantriidae: one species; Bombycidae: two species), Geometridae (one species), Tortricidae (one species), and Plutellidae (one species) (Figs 2 and 5). These PBANs are expected to functionally stimulate pheromone production in different moths, as well as their own species. Although the other PBAN family peptides found in honeybees and beetles are not characterized functionally, the phylogenic relationships of all known PBAN genes were analyzed in the present study. The phylogenetic analysis of PBAN genes matches the phylogeny based on the family level of classification and evolutionary diversity in Insecta, indicating that the neuropeptide sequences could be applied to study insect phylogenetic relationships (Fig. 5). From the PBAN phylogenetic relationship, the fire ant is closest to the honeybee within Hymenoptera followed by the beetle (Coleoptera), and then moths (Lepidoptera). The amino acid sequences from PBAN genes for *So. invicta* and *A. mellifera* showed 56% identical, which decreases to about 30% when compared to lepidopteran moths. Fire ant PBAN, Soi-PBAN (26-AA), is significantly shorter than the PBAN

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S. invicta     1:MI---VTRNSVNRATIV--CIMAMLLCLGSRASGEYESREIGSNGGSSESRSPSNDFGSC  55
A. melifera    1:MIGFAVFS-SFNRFTTIFVCVLLCVVYLLSYASGEYDGRDSSSGSNN-D-RAPSNEFGSC  57
                  **   *   * ** **   *       * * *****  *   *       * *** ****
S. invicta    56:IDGKCIKRTSQDIASGMWFGPRLGKRYKSDEKQELSSEIEILANALDGVRWAVITIPASD 115
A. melifera   58:TDGKCIKRTSQDITSGMWFGPRLGRRRRADRKPEINSDIEAFANAFEEPHWAIVTIPETE 117
                  ************ ********** *   * * *  * **  ***     **  ***
S. invicta   116:KRQ-PQFTPRLGRGSGED-LSYG---DAYEV-DEDD-H-PLFVPRLGRRLPWIPSPRLGR 167
A. melifera  118:KRQITQFTPRLGRESGEDYFSYGFPKDQEELYTEEQIYLPLFASRLGRRVPWTPSPRLGR 177
                  ***  ******** ****  ***   *  *   *     ***  ***** ** *******
S. invicta   168:QLRNVLR---------KL                                          176
A. melifera  178:QLHNIVDKPRQNFNDPRF                                          195
                  ** *
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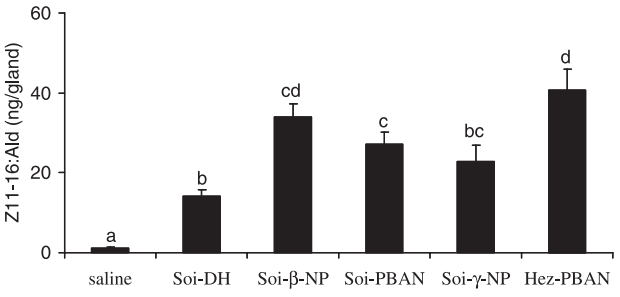
Figure 3. Similarity of PBAN genes from two hymenopteran species, *Solenopsis invicta* (present study) and *Apis mellifera* (GenBank Accession number: NM_001 110 712). The fire ant PBAN gene is about 56 % similarity with honeybee. Identical amino acids in two sequences are indicated with asterisks. Dashed lines indicate gaps.

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Soi-DH  :                TSQDIASGMWFGPRL
Soi-β-NP:                      QPQFTPRL
Soi-PBAN: GSGEDLSYGDAYEVDEDDHPLFVPRL
Soi-γ-NP:                      LPWIPSPRL
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Figure 4. Pheromonotropic activity of synthetic peptides deduced from Soi-PBAN cDNA and Hez-PBAN in *Helicoverpa zea* female moths (top) and four synthetic peptide sequences (bottom). *Bars* represent the means SEM of at least 5 replications. *Bars* with the same *letters* are not statistically different by analysis of Fisher PLSD (ANOVA) ($P < 0.05$).

domain (33-AA) of *A. mellifera* and they share only 35% identity, mostly in their C- and N-terminal regions. However, the other three neuropeptide homologues (Soi-DH, β and γ) have similar sequences to the analogous peptides found in the honeybee.

The shortest and well conserved neuropeptide, α-NP (VIFTPKL), sometimes referred to as PBAN-encoding gene Neuropeptide-7 (PGN-7) is found in all moth PBAN genes and is similar to leucopyrokinin peptides, however, this peptide is not encoded from Soi-PBAN cDNA (Figs 1 and 2). Similarly, this peptide domain is also absent from honeybee and beetle PBAN genes (Hummon *et al.*, 2006; Li *et al.*, 2008). Leucopyrokinin is known to stimulate the contraction of cockroach hindgut muscles (Holman *et al.*, 1986), but it is not known if the peptide plays the same role in lepidopteran moths. Soi-β-NP (8-AA), QPQFTPRL, is relatively shorter than β-NPs found in moths, and is more similar to leucopyrokinin (PETSFTPRL) because Glutamine (Q), Proline (P), Serine (S) and Threonine (T) belong to the same polar and uncharged amino acid residues. To date, all β-NPs discovered from insects contain the FTPRL epitope at their C-termini (Fig. 2). The pheromonotropic activity was highest for Soi-β-NP and was not significantly different from *H. zea*'s own PBAN (Hez-PBAN, Fig. 4). Half-maximum effective concentration (EC_{50}) values of the *H. zea* moth α- and β-NPs binding the PBAN receptor were similar, and active at lower concentrations than any other FXPRL peptides (Choi *et al.*, 2003). The previous and current results indicate that the C-terminal ends of α-NP (FTPKL), β-NP (FTPRL) and PBAN (FSPRL) could have similar conformational structures when bound to the Hez-PBAN receptor. This can be explained in that different amino acids are substituted, but they belong to the same functional family at the C-termini (e.g., Serine (S) and Theronine (T) belong to the polar and uncharged R group, and Lysine (K) and Arginine (R) to the non-polar and aromatic R group). Also, the lack of an α-NP in fire ants could be explained if the domains for α-NP and β-NP in the fire ant were fused together to form a single peptide (Soi-β-NP). The Soi-β-NP (QPQFTPRL) of the fire ant is similar in structure to Neb-pyrokinin-2, SVQFKPRL, identified as a flesh fly pupariation factor, which is known to accelerate pupariation (Verleyen *et al.*, 2004). But, the physiological role of Soi-β-NP in fire ant remains to be determined. The low pheromone production induced by Soi-PBAN and Soi-γ-NP in the moth compared to Soi-β-NP could be due to a non-polar aliphatic R group, Valine (V), positioned in FVPRL of the C-terminus instead of Serine (S), and Proline (P) positioned in PSPRL instead of Phenylalanine (F).

The putative Soi-DH homologue (TSQDIASGMWFGPRL) positioned in the first cleaved domain of Soi-PBAN cDNA, 15-AA, corresponds to the PBAN-encoding gene neuropeptide-24 (PGN-24), a diapause hormone (DH) found in lepidopteran moths. This peptide, with a conserved

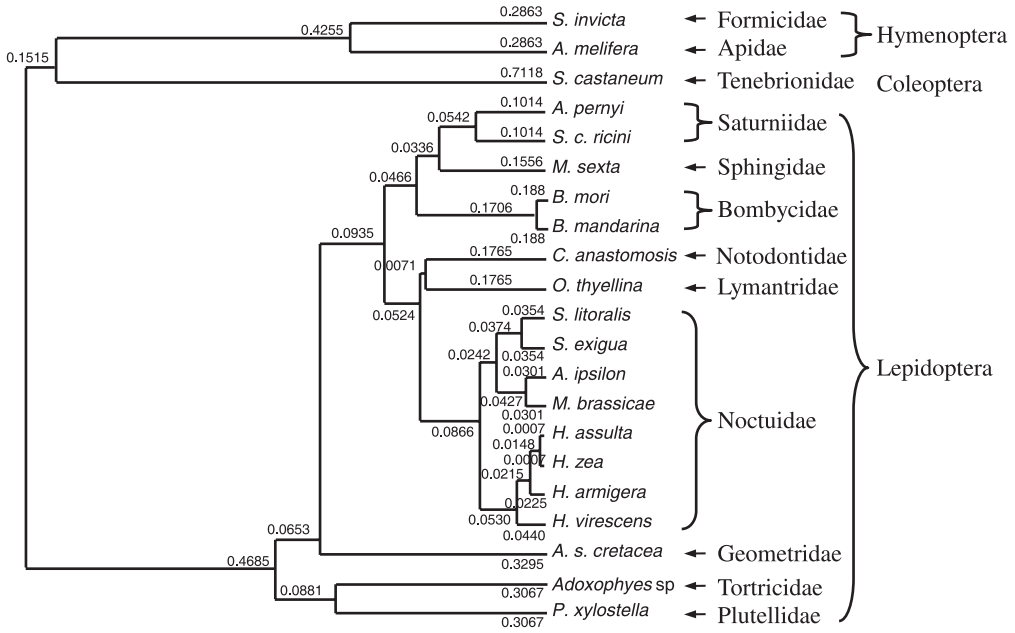

Figure 5. Phylogenetic tree based on full or partial amino acid sequences (= open reading frame) deduced from PBAN cDNAs of different species as shown in Figure 2. The amino acid sequences from *Lymantria dispar* (33-AA) and *Plutella separata* (18-AA) as shown in Figure 2 were not included in this tree due to insufficient number of amino acid sequences for this comparison. The tree was made with a fixed distance scale using the UPGMA method of Genetyx 6.0 software and multiple alignments of amino acid sequences. The numbers indicate phylogenetic distance values in the analysis.

WFGPRLamide sequence at the C-terminal end, was initially identified from the silkworm, where it induced embryonic diapause in *B. mori* (Imai *et al*., 1991), but the function of this peptide is unknown in other moths. This type of peptide, however, has been found in the PBAN/Pyrokinin family of peptides identified from many insect groups indicating that the peptide could be involved in a common function related to insect development including metamorphosis. Recently, the peptide motif has been demonstrated to terminate pupal diapause in heliothine moths (Sun *et al*., 2003; Xu & Denlinger, 2003). The DH type peptide, with a conserved WFGPRLamide sequence at the C-terminus, showed the lowest pheromonotropic activity in the fire ant (this study), and the lowest activity (highest EC_{50}) of the expressed PBAN receptor when compared to the four other *H. zea* FXPRL peptides (Choi *et al*., 2003). These results imply that the WFGPRLamide structure does not bind effectively with the PBAN receptor. The function(s) of the PBAN/pyrokinin family of peptides identified from fire ants has yet to be determined. However, from the previous fire ant PBAN immunocytochemical study (Choi *et al*., 2009), PBAN/pyrokinin family peptides are produced from neurosecretory cells in the SG and abdominal ganglia of all fire ant adult forms and released into the hemolymph. This indicates that these peptides are likely involved in currently unknown physiological event(s) in fire ant adults.

In conclusion, we identified *So. invicta* PBAN/pyrokinin cDNA that encodes four putative peptides, Soi-DH, Soi-β-NP, Soi-PBAN and Soi-γ-NP. The cDNA sequence is phylogenetically distant from lepidopteran PBAN/pyrokinin sequences. Moth PBAN/pyrokinin genes also code for an α-NP homologue, VIFTPKLamide, which is highly conserved, but is absent from the fire ant PBAN/pyrokinin cDNA. Fire ant PBAN (Soi-PBAN) is a relatively short peptide with a low degree of similarity to PBANs identified from other insects. All peptides from Soi-PBAN cDNA have pheromonotropic activity in a moth model, but their own function remains to be elucidated. Based on amino acid sequences encoded from insect PBAN genes identified, neuropeptide hormone diversity is correlated with basic taxonomical or phylogenetic classification of Insecta. This is the first neuropeptide identified and characterized from the central nervous system of Formicidae.

Experimental procedures

Insects

Fire ants. Field collected monogyne fire ant colonies, *Solenopsis invicta,* were collected from Gainesville area, Florida, USA, and maintained at room temperature in the laboratory using standard procedures described previously (Banks *et al*., 1981). The fire ant brain-subesophageal ganglion (Br-SG) was dissected from female alates, and used to isolate mRNA, as described below.

Moths. Pupae of Corn Earworm (*Helicoverpa zea*) moths were shipped from North Carolina State University (Department of Entomology) and maintained at room temperature under L/D regimen of 15:9 until they emerged as adults. Virgin female adults

were decapitated at ca. 24-h early in their second or third photophase and were used throughout this study.

Peptides

Synthetic Soi-PBAN and three fire ant neuropeptides (Sigma Genosys, The Woodlands, TX, USA) and Hez-PBAN (Peninsula Laboratories, San Carlos, CA, USA) were each dissolved in lepidopteran saline (21 mM KCl, 12 mM NaCl, 3 mM $CaCl_2$, 18 mM $MgCl_2$, 85 mM trehalose and 5 mM pipes adjusted to pH 6.6 with KOH), and evaluated for pheromonotropic activity.

Poly (A) RNA isolation and cDNA synthesis

One hundred brain-subesophageal ganglia (Br-SGs) were dissected from fire ant female alates in an autoclaved cold hymenopteran saline (130 mM NaCl , 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, and 10 mM HEPES, adjusted to pH 7.2 with NaOH) and stored at –80 °C until use. Poly $(A)^+$ RNA was isolated from the dissected Br-SGs by Micro Fast mRNA purification kit (Invitrogen, Carlsbad, CA, USA), and used to synthesize cDNA with the GeneRacer cDNA synthesis kit (Invitrogen).

Molecular cloning and characterization

The synthesized cDNA above was amplified with a degenerate primer set: a sense primer, 5′-GGNATGTGGTTYGGNCCNMGNYTNGGNMG-3′ and antisense primer, 5′-CKNCCNA RNCKNGGNCCRAACCACATNCC-3′ based on PBAN cDNA conserved sequences deduced from other PBAN genes, using a PCR-based method. PCR was performed with the following temperature program: 5 cycles at 95 °C for 30 s, 67 °C for 30 s, and 72 °C for 1 min, 5 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, and 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The PCR product was gel purified and cloned using TOPO TA cloning kit (Invitrogen) and sequenced. Based on this partial sequence, the further identification of the full cDNA of Soi-PBAN was performed using gene specific primers indicated with arrows (Fig. 1); sense primer, 5′-ATCGCCAGCGGCATGTGGTTCGG CC-3 for 3′-RACE and antisense primer, 5′-GCACGAATAATGGATGGTCGTC TTC-3′ for 5′-RACE with GeneRacer kits (Invitrogen). The RACE PCR products were ligated into the PCR2.1 vector from the TOPO TA cloning kit (Invitrogen) for sequencing. The obtained full-length sequence information was aligned and sequences compared with our partial sequence using Genetyx DNA software (Genetyx Co., Tokyo, Japan).

Pheromonotropic activity

Synthetic fire ant PBAN (Soi-PBAN) or other peptides deduced from the Soi-PBAN cDNA, or Hez-PBAN were dissolved in lepidopteran saline (1 pmol/μl), and injected (3 μl) between the fourth and the fifth abdominal segments of decapitated *H. zea* females during mid-photophase. The *H. zea* pheromone glands were dissected after 0.5–1 h incubation at room temperature and extracted with hexane containing (Z)-9-tetradecenal (100 ng) as an internal standard. A GC 6890N (Agilent Technologies) equipped with a capillary column (30 m × 0.25 mm, DB-23, J&W) was used to measure the amount of pheromone. The oven temperature was programmed at 80 °C for 1 min, then 10 °C/min to 230 °C and held for 8 min. The results were analyzed by non-parametric analysis as ranks (Fisher PLSD, ANOVA) using STATVIEW 5.0 software.

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