Eur. J. Entomol. **98**: 159–165, 2001
ISSN 1210-5759

Identification of PBAN-like immunoreactivity in the neuroendocrine system and midgut of *Dysdercus cingulatus* (Heteroptera: Pyrrhocoridae)

VISWANATHAN SARADA AJITHA and DAMODARAN MURALEEDHARAN

Department of Zoology, University of Kerala, Kariavattom,
Thiruvananthapuram-695 581, Kerala, India.

Key words. Polyclonal antiserum, Pheromone Biosynthesis Activating Neuropeptide, immunohistochemistry, neuroendocrine system, midgut, *Dysdercus cingulatus*

Abstract. Polyclonal antibodies against PBAN were used to map the distribution of PBAN-like antigenic sites in the brain-suboesophageal ganglion (Br-SOG) complex, associated neurohaemal structures, ventral nerve cord ganglia and in the alimentary canal. A pair of lateral neurosecretory cells immunopositive to the antiserum were found in each half of the deutocerebrum. PBAN-like immunoreactivity (PLI) was also noticed in the tritocerebral region of the brain and in the aorta. Two groups of immunopositive cells of four and two cells respectively, were found in the SOG. Small and weakly immunoreactive neurons were identified in the prothoracic ganglion, whereas in the pterothoracic ganglion a pair of cells reacted positively to the antibody. Immunoreactive cells were also identified in the corpora cardiaca. Some of the epithelial endocrine cells of the midgut also showed immunoreactivity to PBAN antiserum.

INTRODUCTION

Pheromone Biosynthesis Activating Neuropeptide (PBAN) is reported to be involved in the regulation of pheromone production in many lepidopteran insects (Raina, 1993). This factor consists of a 33-aminoacid polypeptide with an amidated carboxyl terminal and is present in the brain-suboesophageal ganglion (Br-SOG) extracts of *Helicoverpa zea* (Raina et al., 1989), *Bombyx mori* (Kitamura et al., 1989, 1990), *Lymantria dispar* (Masler et al., 1994). Its structure was deduced from cDNA in *Mamestra brassicae* (Jaquin Joly et al., 1998), *Agrotis ipsilon* (Duportets et al., 1998) and *Helicoverpa assulta* (Choi et al., 1998). A high degree of sequence homology exists in these peptides from different species of insects.

PBANs are members of the pyrokinin family of neuropeptides, which are engaged in multiple physiological functions in insects, characterised by a common C-terminal sequence FXPRL amide (Abernathy et al., 1995). The C-terminal pentapeptide motif has been shown to be the shortest pheromonotropic fragment (Raina & Kempe, 1990) required for biological activity. PBAN, diapause hormone and other neuropeptides with the FXPRL-NH_2 C-terminus result from the proteolytic processing of a protein precursor in both *Bombyx mori* and *Helicoverpa zea* (Sato et al., 1993; Ma et al., 1994). According to Matsumoto et al. (1990), the same neuropeptide controls cuticular melanization in larvae and pheromone production in adults of lepidoptera. PBAN or peptide(s) that belong to the PBAN peptide family induce embryonic diapause in *Bombyx mori* (Imai et al., 1991) and have myotropic functions in cockroaches and locusts (Holman et al., 1986; Schoofs et al., 1991).

The neuroendocrine tissues of insects react positively to several vertebrate and invertebrate bioactive peptides (Kramer, 1984). The gut is also a very important endocrine organ in insects as a number of bioactive peptides have been immunochemically demonstrated there (Sehnal & Zitnan, 1990). Midgut and hindgut of insects receive granule-filled axons from the last abdominal ganglion (Nayar, 1954; Brown, 1967) and from corpora cardiaca directly (Johnson & Bowers, 1963).

The presence of immunoreactivity in several species of lepidopteran insects suggests a wide distribution of PBAN-like peptides within this order. However, pheromonotropic activity has also been found in the heads of other non-lepidopteran insects, such as cockroaches and locusts (Sreng & Roelofs, 1988; Sreng et al., 1990). This activity could be due to the presence of PBAN or neuropeptides that share the active C-terminal part of the molecule with PBAN (Fonagy et al., 1992; Kuniyoshi et al., 1992). *Dysdercus cingulatus* is a serious pest of cotton and there is no evidence of this family of neuropeptides in heteropteran insects. Hence it was thought reasonable to look for PBAN-like peptides in this particular insect, which might motivate further research leading to the control of this pest insect.

MATERIALS AND METHODS

Insects

The insect used in this study, *Dysdercus cingulatus* (Heteroptera: Pyrrhocoridae) was reared in our laboratory under controlled conditions (temp. 28 ± 3°C, cycle 12L : 12D and r.h. 90 ± 3) and fed on soaked cotton seeds.

Antisera production

Polyclonal antibodies to synthetic *H. zea* PBAN (Peninsula Lab Inc., Belmont. CA) were raised in New Zealand strain of white rabbit. The rabbit was immunised with 250 µg of PBAN in Freund's complete adjuvant injected subcutaneously. Ten days later another booster injection was given of the same

amount of PBAN in Freund's incomplete adjuvant. On 20th day, an additional booster dosage of 250 µg of antigen in 500 µl PBS was injected. Seven days later the rabbit was bled from the marginal ear vein. The serum was separated by centrifugation, aliquoted and stored at –40°C. The antiserum was further subjected to affinity purification to improve its specificity. For this the method followed was that of Kingan et al. (1992).

Dissection and fixation

Female insects of different ages were used. The brain-SOG with associated retrocerebral complex (CC-CA and aorta) and prothoracic and pterothoracic ganglia were dissected under ice-cold insect saline (Bindokas & Adams, 1988). The gut was fixed with a droplet of Bouin's fixative containing saturated picric acid, formaldehyde and glacial acetic acid and gut contents were removed. For paraffin sections, the tissues were embedded in paraffin wax with ceresin (MP. 60°C). Sections were cut at 6µm thickness and mounted on albumin-coated glass slides. For wholemount immunostaining, tissues were fixed in Bouin's fixative for 2 hrs at room temperature.

Immunohistochemistry

For immunostaining of paraffin sections, the two step immunoperoxidase method of Schooneveld & Veenstra (1988) was followed. The sections were deparaffinised in xylene, then rehydrated and the activity of endogenous peroxidase blocked by immersing for 20 min in 0.05% hydrogen peroxide in PBS and treated with 10% normal goat serum for 10 min. Sections were incubated overnight at 4°C in antiserum against Hez-PBAN at different dilutions such as 1 : 500, 1 : 1000, 1 : 1500, 1 : 2000 and 1 : 2500. Slides were again rinsed in PBS, then treated with the secondary antibody (peroxidase labelled goat-anti rabbit Ig from Bangalore Genei, India) at a dilution of 1 : 100 for 1 hr at room temperature. The sections were then rinsed again in PBS, stained with a mixture of PBS, H_2O_2 and 3-3′-diaminobenzidene tetra hydrochloride (DAB-HCl), dehydrated in ethanol series, cleared in xylene and mounted in DPX. The same experiments were conducted using the Hez-PBAN antiserum (a gift from Dr. A.K. Raina, USA) at a dilution of 1 : 2500.

Wholemount immunostaining

After fixation, tissues were washed with cold 70% ethanol overnight, followed by washes in 70% ethanol, 95% ethanol with 0.1% H_2O_2 and 70% ethanol, and then rinsed several times in cold PBS containing 1% Triton X-100 (PBST), until the yellow colour was completely removed. Tissues were then pre-incubated in blocking serum, washed and incubated with primary antiserum for 24 hrs at 4°C. After washing with cold PBST, the tissues were incubated with secondary antibody for 1 hr at room temperature. After the final washing in cold PBS and once in 0.05 M Tris-HCl (pH 7.6), the tissues were stained with Tris-HCl containing 0.05% DAB and 0.03% H_2O_2 for 10 minutes. The staining was stopped by rinsing in PBS, and then the tissues were dehydrated, cleared and mounted.

Control slides were prepared by:

i. Omission of primary antibody to check nonspecific binding of secondary antibodies and other compounds applied in the procedure.
ii. Replacing specific antisera with pre-immunoserum.
iii. Pre-adsorption of antiserum overnight with 0.001 mg/ml Hez-PBAN.

RESULTS

These studies utilized two different polyclonal antisera. Different dilutions of our primary antisera were checked and the best results were obtained at a dilution of 1 : 500. When the antiserum provided by Dr. A.K. Raina was used the dilution was 1 : 2500, as recommended. Both antisera revealed the same PLI in all these structures.

Immunostaining of the brain revealed one pair of PBAN-positive lateral neurosecretory cells in the deutocerebrum (Fig. 1). PBAN-like immunoreactivity could be localised in the tritocerebral region of the brain and considerable amount of PLI was also detected in the wall of the aorta (Fig. 2). Serial sections of the Br-SOG complex revealed two clusters of PBAN-like immunoreactive cells within the SOG with four and two cells in each group (Fig. 3, 4, 5). Horizontal sections revealed the presence of immunoreactive neurons in the CC (Fig. 6). Corpus allatum (CA) showed no detectable amount of immunoreactivity.

A few stained neurons were found to be present in the prothoracic ganglion. However staining of these neurons was generally weak and disappeared when a higher dilution of the antiserum (1 : 2500) was used. In this group of insects, the mesothoracic, metathoracic and abdominal neuromeres fuse to form a structure called the 'pterothoracic ganglion'. One pair of lateral immunopositive somata was observed in this ganglion (Fig. 9).

There was very little PBAN-like immunoreactivity in newly emerged adults. But after 48 hours, i.e. before they started mating, the immunoreactive cells contained large quantities of neurosecretion. However, after mating, PLI could not be detected in these tissues (Fig. 7, 8).

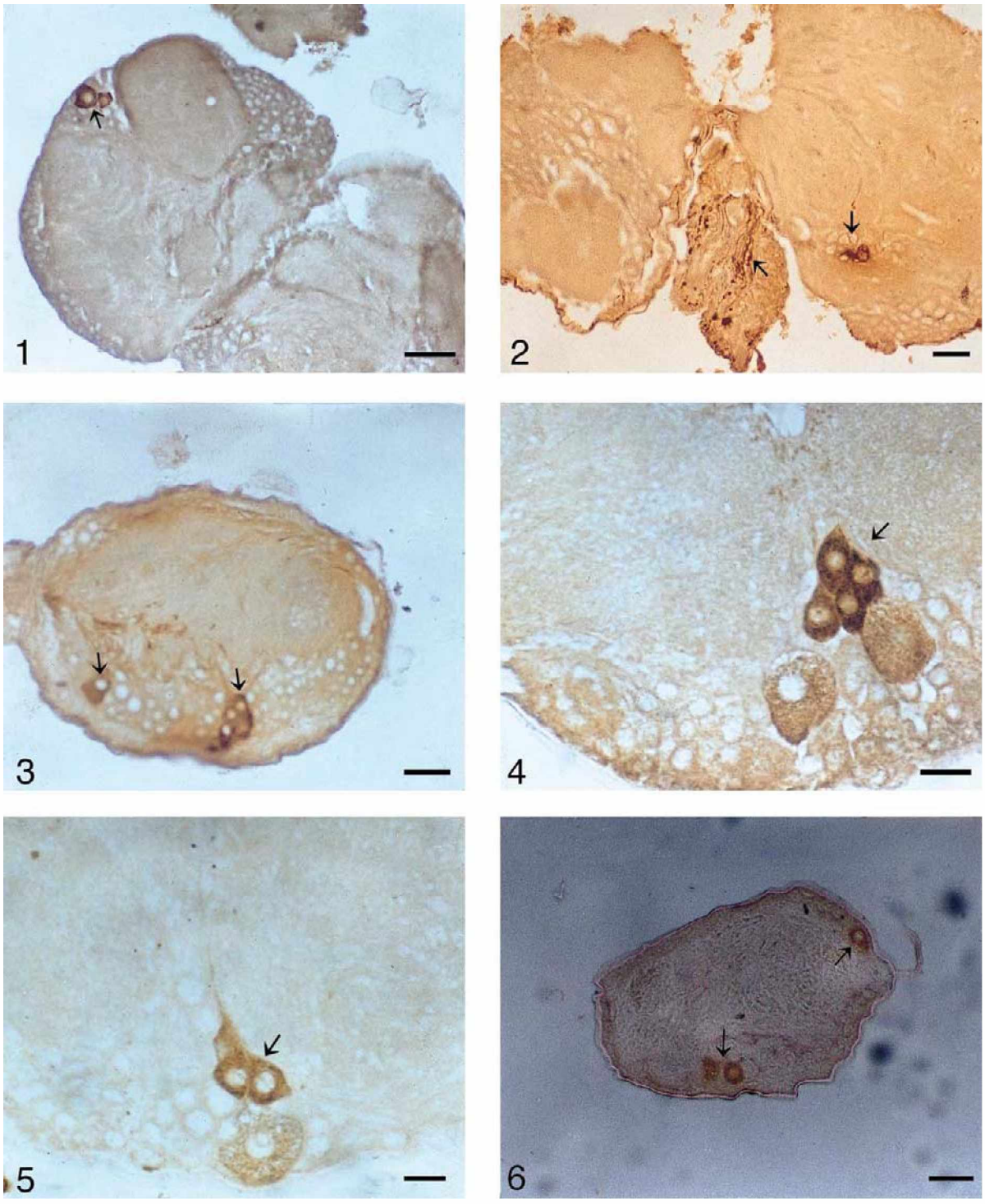
In this insect the midgut is composed of four regions, the first, second, third and fourth ventriculi. PLI was detected in the first and second ventriculus of the midgut (Fig. 10, 11). Some of the epithelial endocrine cells showed an immunopositive reaction to the antiserum (Fig. 11).

No immunostaining was observed when pre-immune serum was used and PBAN-like immunoreactivity was absent when the antiserum was preadsorbed overnight with Hez-PBAN.

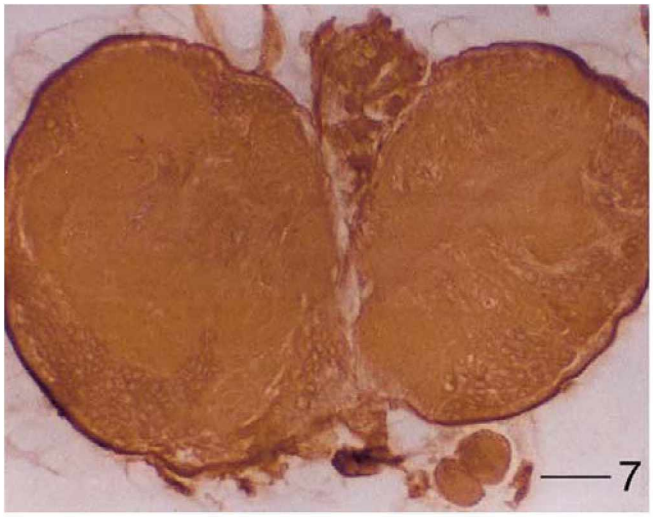
DISCUSSION

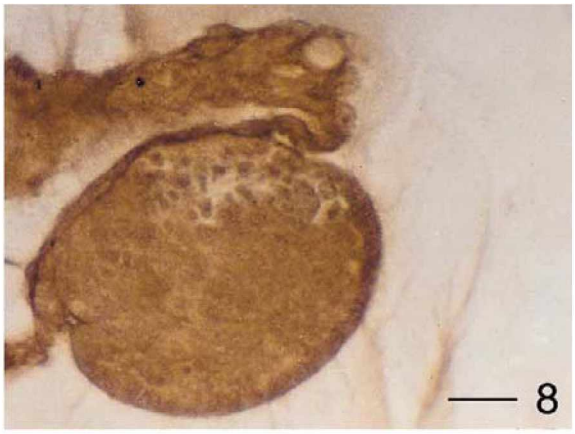
Pheromone production in lepidopteran insects is known to be regulated by the neuropeptide PBAN. We used an antiserum to Hez-PBAN to map the distribution of immunoreactive somata in the neuroendocrine system as well as the alimentary canal of the heteropteran insect *Dysdercus cingulatus*.

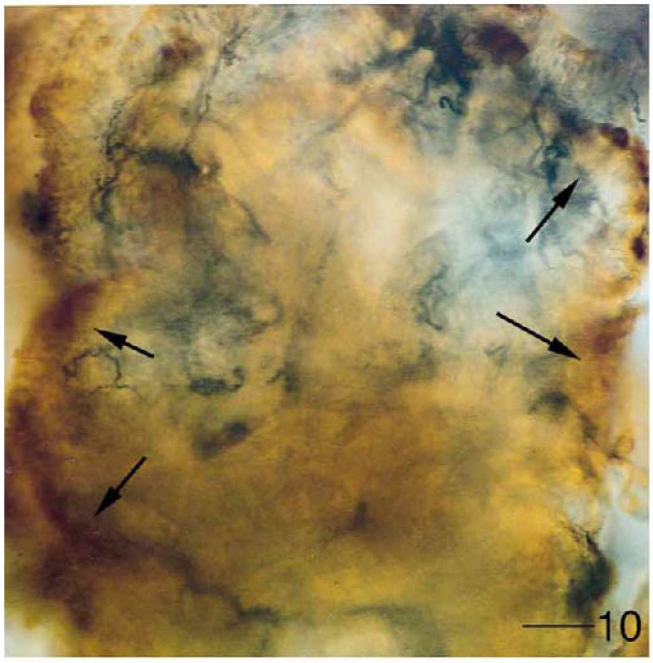
Immunohistochemical studies revealed a pair of PBAN positive lateral neurosecretory cells in each half of the deutocerebrum. The presence of PLI in the tritocerebral region and the aorta suggests neurohaemal function of aotra. This finding contradicts the results obtained with other lepidopteran insects, where PLI was not detected in the brain (Blackburn et al., 1992; Ichikawa et al., 1995; Davis et al., 1996). However, Kingan et al. (1992) identified a fine perhaps 'fuzzy' immunoreactivity in the tritocerebrum of *Helicoverpa zea*. They suggested that this tritocerebral staining is of branches of the maxillary neurites near their dorsal extension in the SOG.

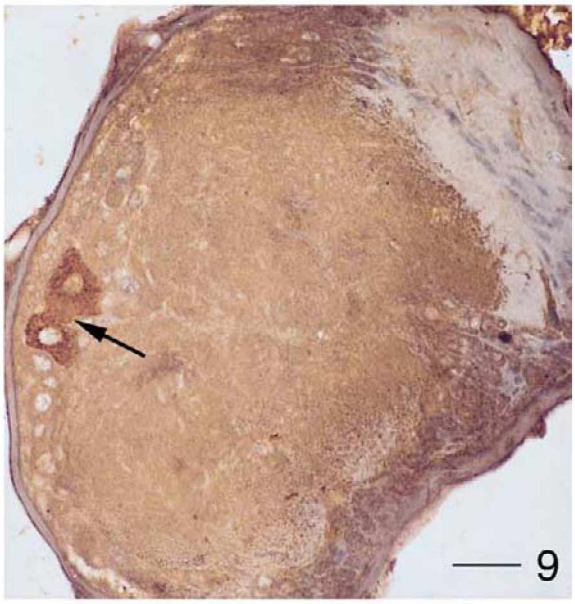


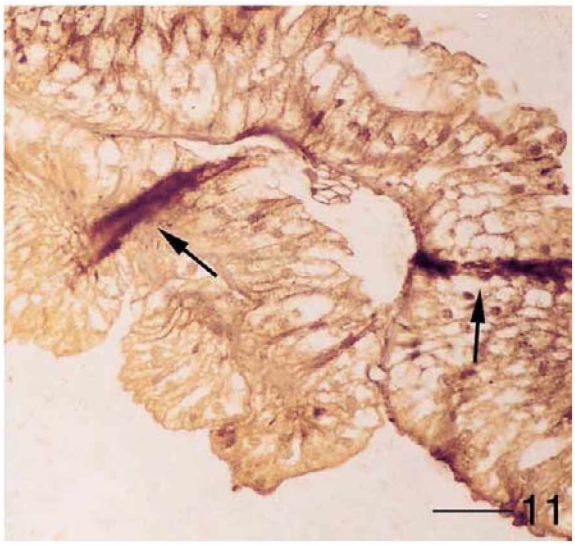
Figs 1 – 6: 1 – sagittal section of the brain showing PBAN-like immunoreactive cells in the lateral region of the deutocerebrum (arrow mark); 2 – tritocerebral immunopositive neurons and their extension to the aorta; 3 – two groups of maxillary and labial immunopositive somata in the SOG; 4 – 5 – enlarged view of the two sets of somata in the SOG. Scale 25 μm; 6 – immunoreactivity exhibited by CC. Scale 40 μm.











Figs 7 – 11: 7 – section of brain and CC showing lack of immunoreaction after mating. Scale 25 µm; 8 – section of brain and CC showing lack of immunoreaction after mating. Scale 40 µm; 9 – immunopositive somata in the pterothoracic ganglion; 10 – immuno-wholemount of the first ventriculus of the midgut showing PBAN-like immunoreactivity; 11 – L.S. of second ventriculus showing immunoreactive endocrine cells. Scale 40 µm.

Two clusters of immunoreactive somata were detected in the SOG of *Dysdercus cingulatus*. This also differs from that reported for several lepidopteran insects like *Helicoverpa zea* (Blackburn et al., 1992; Kingan et al., 1992), *Bombyx mori* (Ichikawa et al., 1995), *Manduca sexta* (Davis et al., 1996), *Lymantria dispar* (Golubeva et al., 1997) and recently for *Agrotis ipsilon* (Duportets et al., 1998) where there are three clusters of cells – the mandibular, maxillary and labial. The insect SOG is formed by the fusion of the neuromeres of the mandibular, maxillary and labial segments. In this study maxillary and labial neuroendocrine cells showed PBAN-like immunoreactivity. Golubeva et al. (1997) have shown that many of the SOG cells in *L. dispar* are neurosecretory, in that they have varicose endings in identified neurohaemal organs. In *D. cingulatus* the immunoreactivity found in the CC may indicate its functions as a neurohaemal structure for the peptide.

In this study PLI was weak in the prothoracic ganglion but a pair of large neurosecretory cells was located in the pterothoracic ganglion. In the gypsy moth, the prothoracic ganglion and pterothoracic ganglion each contain a pair of ir-somata (Golubeva et al., 1997). A pair of ventral midline cells in each of the abdominal ganglion including the terminal one (TAG) are immunopositive in the European corn borer (Ma & Roelofs, 1995).

There is now considerable evidence of both central and peripheral sites of action for various peptidergic neurons in insects (Nassel, 1995). The patterns of staining revealed by both immunocytochemistry and ELISA bioassays indicated that there are sites of release not only into the haemolymph, but also within the CNS itself (Rafaeli et al., 1991; Kingan et al., 1992; Davis et al., 1996). Thus activation of the pheromone glands in lepidopterans may involve both neural and hormonal mechanisms. In contrast to the pheromone glands in lepidoptera, the function is carried out by the scent glands in heteroptera (Staddon, 1979).

Cross biological activities are often observed between the FXPRL amide peptides of which PBAN is a member. Further research is therefore needed to confirm that these immunoreactive cells are engaged only in pheromone production in this heteropteran insect. The high immunoreactivity observed before mating and lack of immunoreaction after mating suggest a possible role in eliciting mating behaviour.

PBAN-like material was also observed in different regions of the alimentary canal, especially in the midgut of *D. cingulatus*. This distribution of PBAN-like immunoreactivity suggests a multiplicity of functions besides the regulation of pheromone production. Yu et al. (1995) observed an allatostatin immunoreactivity in the proctodeal innervation and endocrine cells of the midgut, which was previously demonstrated in neurosecretory cells of the brain and ventral nerve cord ganglia of *D. punctata*. These peptides were identified originally on the basis of their ability to inhibit JH biosynthesis by CA. They were subsequently found to inhibit myogenic contractions of the hindgut in cockroaches. FMRF-amide positive cells of the abdominal ganglion send axons *via* the median abdominal nerve to the hindgut where they form an innervation pattern very similar to that of proctolin-containing neurons (An et al., 1998). In *M. sexta*, eclosion hormone containing neurons have varicose axons running through the entire length of cephalic, thoracic and abdominal ganglia before they reach their release sites on the hindgut (Truman & Copenhaver, 1989).

Bom-PBAN gene codes for four other insect neuropeptides, which regulate pheromone production, induction of embryonic diapause, melanization of the integument and also stimulate muscle contraction in insect gut (Kawano et al., 1992). These peptides share with PBAN the C-terminal pentapeptide FXPRL-NH_2. Bom-PBAN I can produce a strong melanization of the cuticle of *Pseudaletia separata* (Matsumoto et al., 1990).

Thus, it seems that PBAN-like immunoreactivity exhibited by epithelial endocrine cells of the gut could result from cross-reactivity of the PBAN-antiserum with other unidentified myotropic peptides of the same family, which share the active C-terminus of the peptide. There is evidence that some gut endocrine cell products enter the body circulation and exert hormonal effects on distal targets. Due to the various routes of action, a single hormone can exert a variety of effects in insects (Sehnal & Zitnan, 1996). Further research is however needed to confirm the functions of PBAN-like peptides in this group of insects.

ACKNOWLEDGEMENTS. The research was supported by the grants-in-aid from the Council of Scientific and Industrial Research, India (No : 37(0860) 94 EMR – II). We are grateful to Dr.A.K.Raina, USDA for the generous supply of Hez-PBAN antibody to confirm our results.

REFERENCES

ABERNATHY R.L., NACHMAN R.J., TEAL P.E.A., YAMASHITA O. & TUMLINSON J.H. 1995: Pheromonotropic activity of naturally occurring pyrokinin insect neuropeptides (FXPRL amide) in H. zea. *Peptides* **16**: 215–219.

AN Y., NAKAJIMA T. & SUZUKI K. 1998: Immunohistochemical demonstration of mammalian- and FMRF amide-like peptides in the gut innervation and endocrine cells of the wild silkmoth, A. yamamai, during diapause and post diapause of pharate first instar larvae. *Eur. J. Entomol.* **95**: 185–196.

BINDOKAS V.P., ADAMS M.E. 1988: Haemolymph composition of the tobacco budworm H. virescens F. *Comp. Biochem. Physiol.* **90**: 151–155.

BLACKBURN M., KINGAN T.G., RAINA A.K. & MA M.C. 1992: Colocalization and differential expression of PBAN- and FMRF amide-like immunoreactivity in the suboesophageal ganglion of H. zea (Lepidoptera: Noctuidae) during development. *Arch. Insect Biochem. Physiol.* **21**: 225–238.

BROWN B.E. 1967: Neuromuscular transmitter substance in insect visceral muscle. *Science* **155**: 155–595.

CHOI M.Y., TANAKA S., KATAOKA H., BOO K.S. & TATSUKI S. 1998: Isolation and identification of the cDNA encoding the pheromone biosynthesis activating neuropeptide and additional neuropeptides in the oriental tobacco budworm H. assulta (Lepidoptera: Noctuidae). *Insect Biochem. Mol. Biol.* **28**: 759–766.

DAVIS N.T., HOMBERG U., TEAL P.E.A., ALTSTEIN M., AGRICOLA H.J & HILDEBRAND J.G. 1996: Neuroanatomy and immunocy-

tochemistry of the median neuroendocrine cells of the suboesophageal ganglion of the tobacco hawk moth, M.sexta: Immunoreactivities to PBAN and other neuropeptides. *Microsc. Res. Tech.* **35**: 201–229.

DUPORTETS L., GADENNE C., DUFOUR M.C. & COUILLAUD F. 1998: Pheromone Biosynthesis Activating Neuropeptide (PBAN) of black cutworm moth, Agrotis ipsilon: Immunochemistry, molecular characterization and bioassay of its peptide sequence. *Insect Biochem. Mol. Biol.* **28**: 591–599.

FONAGY A., SCHOOFS L., MATSUMOTO S., DE LOOF A. & MITSUI T. 1992: Functional cross-reactivities of some Locusta myotropins and Bombyx PBAN. *J. Insect Physiol.* **38**: 651–657.

GOLUBEVA E., KINGAN T.G., BLACKBURN M.B., MASLER E.P. & RAINA A.K. 1997: The distribution of PBAN-like immunoreactivity in the nervous system of the gypsy moth, Lymantria dispar. *Arch. Insect Biochem. Physiol.* **34**: 391–408.

HOLMAN G.M., COOK B.J. & NACHMAN R.J. 1986: Isolation, primary structure and synthesis of two neuropeptides from Leucophaea maderae: members of a new family of cephalomyotropins. *Comp. Biochem. Physiol.* **84**: 205–211.

ICHIKAWA T., HASEGAWA K., SHIMIZU I., KATSUNO H., KATAOKA H. & SUZUKI A. 1995: Structure of neurosecretory cells with immunoreactive diapause hormone and Pheromone Biosynthesis Activating Neuropeptide in the silkworm, B.mori. *Zool. Sci.* **12**: 703–712.

IMAI K., KONNO T., NAGASAWA Y., KOMIYA T., ISOBE M., KOGA K., SAKAKIBARA K., HASEGAWA K. & YAMASHITA O. 1991: Isolation and structure of diapause hormone of the silkworm, B.mori. *Proc. Japan Acad. Ser. B.* **67**: 98–101.

JACQUIN-JOLY E., BURNET M., FRANCOIS M.-C., AMMAR D., MEILLOUR P.N.-L. & DESCOINS C. 1998: cDNA cloning and sequence determination of pheromone biosynthesis activating neuropeptide of M.brassicae: a new member of the PBAN family. *Insect Biochem. Mol. Biol.* **28**: 251–258.

JOHNSON H. & BOWERS B. 1963: Transport of neurohormones from the corpora cardiaca in insects. *Science* **141**: 264–266.

KAWANO T., KATAOKA H., NAGASAWA H., ISOGAI A. & SUZUKI A. 1992: cDNA cloning and sequence determination of the Pheromone Biosynthesis Activating Neuropeptide of the silkworm B.mori. *Biochem. Biophys. Res. Commun.* **189**: 221–226.

KINGAN T.G., BLACKBURN M.B. & RAINA A.K. 1992: The distribution of Pheromone Biosynthesis Activating Neuropeptide (PBAN) immunoreactivity in the central nervous system of the corn earworm moth, Heliothis zea. *Cell Tissue Res.* **270**: 229–240.

KITAMURA A., NAGASAWA H., KATAOKA H., INOUE T., MATSUMOTO S., ANDO T. & SUZUKI A. 1989: Aminoacid sequence of PBAN of the silkmoth, B.mori. *Biochem. Biophys. Res. Commun.* **163**: 520–526.

KITAMURA A., NAGASAWA H., KATAOKA H., ANDO T. & SUZUKI A. 1990: Amino acid sequence of PBAN-II of the silkworm moth B.mori. *Agric. Biol. Chem.* **54**: 2495–2497.

KRAMER K.J. 1984: Vertebrate hormones in insects. In Kerkut G.A. & Gilbert L.I. (eds): *Comp. Insect Physiol. Biochem. Pharmacol.* Pergamon Press, Oxford, pp. 511–536.

KUNIYOSHI H., NAGASAWA H., ANDO T., SUZUKI A., NACHMAN R.J. & HOLMAN G.M. 1992: Cross-reactivity between pheromone biosynthesis activating neuropeptide (PBAN) and myotropic pyrokinin insect peptides. *Biosci. Biotech. Biochem.* **56**: 167–168.

MA P.W.K., KNIPPLE D.C. & ROELOFS W.L. 1994: Structural organization of the H.zea gene encoding the precursor protein for pheromone biosynthesis activating neuropeptide and other neuropeptides. *Proc. Natl. Acad. Sci. USA* **91**: 6506–6510.

MA P.W.K. & ROELOFS W.L. 1995: Sites of synthesis and release of PBAN-like factor in female European cornborer, Ostrinia nubilalis. *J. Insect Physiol.* **41**: 339–350.

MASLER E.P., RAINA A.K.,WAGNER R.M. & KOCHANSKI J.P. 1994: Isolation and identification of a pheromonotropic peptide from the brain-suboesophageal ganglion complex of Lymantria dispar: a new member of the PBAN family. *Insect Biochem. Mol. Biol.* **24**: 829–836.

MATSUMOTO S., KITAMURA A., NAGASAWA H., KATAOKA H., ORIKASA C. & SUZUKI A. 1990: Functional diversity of a neurohormone produced by the suboesophageal ganglion: molecular diversity of melanization and reddish colouration hormone and pheromone biosynthesis activating neuropeptide. *J. Insect Physiol.* **36**: 427–432.

NASSEL D.R. 1995: Neuropeptide diversity in the insect nervous system. Facts and speculation. In Muraleedharan D. & Jacob M. (eds): *Recent Advances in Insect Endocrine Research*, AAE, Trivandrum, pp. 1–38

NAYAR K.K. 1954: The neurosecretory system of the fruit fly, C.cucurbitae I. Distribution and description of the neurosecretory cells in the adult fly. *Proc. Indian Acad. Sci. B* **40**: 130–144.

RAFAELI A., HIRSCH J., SOROKER V., KAMENSKY B. & RAINA A.K. 1991: Spatial and temporal distribution of PBAN in H.armigera using RIA and in vitro bioassay. *Arch. Insect Biochem. Physiol.* **18**: 119–129.

RAINA A.K. 1993: Neuroendocrine control of sex pheromone biosynthesis in Lepidoptera. *Annu. Rev. Entomol.* **38**: 329–349.

RAINA A.K., JAFFE H., KEMPE T.G., KEIM P., BLACHER R.W., FALES L.M., RILEY L.T., KLUN J.A., RIDGWAY R.L. & HAYES D.K. 1989: Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science* **244**: 796–798.

RAINA A.K. & KEMPE T.G. 1990: A pentapeptide of the C-terminal sequence of PBAN with pheromonotropic activity. *Insect Biochem.* **20**: 849–851.

SATO Y., OGUCHI M., MENJO N., IMAI K., SAITO H., IKEDA M., ISOBE M. & YAMASHITA O. 1993: Precursor polyprotein for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm, B.mori, characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides. *Proc. Natl. Acad. Sci. USA* **90**: 3251–3255.

SCHOOFS L., HOLMAN G.M., HAYES T.K., NACHMAN R.J. & DE LOOF A. 1991: Isolation, primary structure and synthesis of locusta pyrokinin: a myotropic peptide of L. migratoria. *Gen. Comp. Endocrinol.* **81**: 97–104.

SCHOONEVELD H. & VEENSTRA J.A. 1988: In Gilbert L.I. & Miller T.A. (eds): *Immunological Techniques in Insect Biology*. Springer-Verlag, Berlin, pp.75–93.

SEHNAL F. & ZITNAN D. 1990: In Epple A., Scanes C.G. & Stetson M.H. (eds): *Progress in Comparative Endocrinology*. Wiley-Liss, NewYork, pp. 510–515.

SEHNAL F. & ZITNAN D. 1996: Midgut endocrine cells. In Lehane M.J. & Billingsley P.F. (eds): *Biology of the Insect Midgut*. Chapman & Hall, London, pp. 55–85.

SRENG L. & ROELOFS W.L. 1988: Nerveux neuropeptides de Blatte (Dictyoptera) et de Grillon (Orthoptera), stimulant la production de pheromone chez la Pyrale du mais (Lepidoptera). *Abstr. Annls. Soc. Entomol. Fr.* **24**: p. 453.

SRENG L., MOREAU R. & GIRARDIE A. 1990: Locust neuropeptides stimulating sex pheromone production in female European cornborer moth, Ostrinia nubilalis. *J. Insect Physiol.* **36**: 729–726.

STADDON B.W. 1979: The sent glands of Heteroptera. In Treherne J.E., Berridge M.J. & Wigglesworth V.B. (eds): *Advances in Insect Physiology*. Academic Press, London, pp. 351–418.

TRUMAN J.W. & COPENHAVER P.F. 1989: The larval eclosion hormone neurons in M.sexta: Identification of the brain-proctodeal neurosecretory system. *J. Exp. Biol.* **147**: 457–470.

YU C.G., STAY B., DING Q., BENDENA W.G. & TOBE S.S. 1995: Immunochemical identification and expresssion of allatostatins in the gut of D. punctata. *J. Insect Physiol.* **41**: 1035–1043.

Received January 26, 2000; revised June 8, 2000; accepted January 26, 2001