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Functional Differentiation of Neurosecretory Cells with Immunoreactive Diapause Hormone and Pheromone Biosynthesis Activating Neuropeptide of the Moth, *Bombyx mori*

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ABSTRACT—The suboesophageal ganglion of the silkworm, *Bombyx mori*, contains three clusters of neurosecretory cells that are immunoreactive with antisera against the diapause hormone (DH) and the pheromone biosynthesis-activating neuropeptide (PBAN), the two neurohormones that are generated from a common precursor protein. The cells lie on the ventral midline of the ganglion. Neurosecretory cell clusters responsible for the diapause induction activity and the pheromonotropic activity of females were determined by surgically removing one or two of the three clusters of the DH/PBAN immunoreactive cells. A potent diapause induction activity was obtained in females retaining a posterior cluster of cells while a strong pheromonotropic activity was obtained in case of females with a medial cluster. The functional differentiation of these cells may relate to different biochemical and/or physiological natures.

INTRODUCTION

Diapause hormone (DH), a neurohormone originating from the suboesophageal ganglion (SG) of the silkworm, *Bombyx mori,* acts on the developing ovaries of a pharate adult to induce diapause eggs (Fukuda, 1951; Hasegawa, 1951; Hasegawa, 1957). DH is identified as an amidated peptide consisting of 24 amino acid residues (Imai *et al.*, 1991; Sato *et al.*, 1992). The SG of the silkworm produces another neurohormone, pheromone biosynthesis activating neuropeptide (PBAN) that stimulates the biosynthesis of a sex pheromone (bombykol) in pheromone glands of a female moth to attract the male. Two forms of PBAN molecules have been isolated: PBAN-I consists of 33 amino acid residues and the sequence of PBAN-II is identical to PBAN-I except for the presence of an additional residue at the amino terminus (Kitamura *et al.*, 1989, 1990). Similar neuropeptides have been identified in two other species of moths (Masler *et al.*, 1994; Raina *et al.*, 1989).

DH and PBAN share a common C-terminal pentapeptide amide (FXPRL amide) and they are classed into the same peptide family (Imai *et al.*, 1991; Kitamura *et al.*, 1989). Cloning and sequencing of cDNA encoding DH or PBAN have shown that the cDNA encodes a precursor protein that contains the sequence of PBAN and three other neuropeptides in the region following DH, thereby suggesting that five neuropeptides are generated from a common precursor polyprotein translated from a single species of mRNA (Kawano *et al.*, 1992; Sato *et al.*, 1993).

Twelve neurosecretory cells expressing the gene for the polyprotein precursor are aggregated into three clusters localized at the ventral surface of the mandibular (anterior), maxillary (medial) and labial (posterior) neuromeres of the ganglion (Sato *et al.*, 1994). These neurosecretory cells are immunoreactive to antisera raised against DH and PBAN (Ichikawa *et al.*, 1995). It remains unknown whether all these DH/PBAN immunoreactive cells are responsible for both the DH and PBAN activity.

Evidence has now been obtained that the posterior cells exhibit a potent diapause induction activity while the medial cells possess a strong pheromonotropic activity.

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MATERIALS AND METHODS

The silkworms of a hybrid race, Kinshu x Showa, were used. Eggs were incubated at 25°C under the continuous light in order to obtain the animals destined to produce diapause eggs. Larvae were reared on a synthetic diet and kept at 25°C under a light regime (16L:8D). Pupae and adults were also kept under the same conditions.

Newly ecdysed female pupae were selected and kept in a refrigerator (4°C) overnight to immobilize the pupae. The suboesophageal ganglion (SG) was exposed by making a window on the ventral cuticle of the head. In the first experiment, SG was transected using micro-scissors to remove an anterior half or a posterior half of the SG: the former contained the anterior and medial clusters of the DH/PBAN immunoreactive cells and the latter had the posterior cluster of the cells. In some pupae, SG was removed after transecting the nerve cords connecting SG to the brain and to the first thoracic ganglion.

In the second experiment, somata of all or two particular clusters of the neurosecretory cells were removed using an electrolytically-sharpened tungsten needle after removing part of the neural sheath covering the ventral surface of the SG. Opalescent appearance of the somata aided in identifying them under a dissecting microscope. In sham-operated females, a window was made on the ventral cuticle of the head and wounds were sealed with melted wax.

Pheromone glands and ovaries were excised from females at a midpoint of the second photophase after eclosion, a time when the pheromone titer is maximal (Ando *et al.*, 1988). DH activity was expressed as the amount of 3-hydroxykynurenine in the ovaries, because diapausing eggs accumulate the substance that is a metabolic precursor of ommochrome pigments (Sonobe and Ohnishi, 1970). 3-hydroxykynurenine was extracted and diazo-oxidized as described by Inagami (1954) and the amount was calculated from absorbance at 410 nm. Pheromone titers were determined with HPLC as described by Ando *et al.* (1988).

To verify that surgical operations were successful, the brain-SG or SG-thoracic ganglion complexes were isolated after the pheromone gland and the ovary were excised. The complexes were usually fixed in Bouin's solution, dehydrated, and embedded in paraffin. Cells remaining in the complexes were immunocytochemically visualized, using antisera raised against 15 residues of N-terminal sequence of PBAN (Ichikawa *et al.*, 1995). For wholemount preparations, immunocytochemical visualization was done after fixation (Ichikawa *et al.*, 1995). The immunocytochemical examination revealed that all operations in the first experiment were successful but operations in the second experiment were sometimes incomplete. Data obtained from incompletely operated females were discarded.

The structure of some posterior cells was examined by injecting Lucifer Yellow into the cells, according to the method described previously (Ichikawa, 1991; Ichikawa *et al.*, 1995).

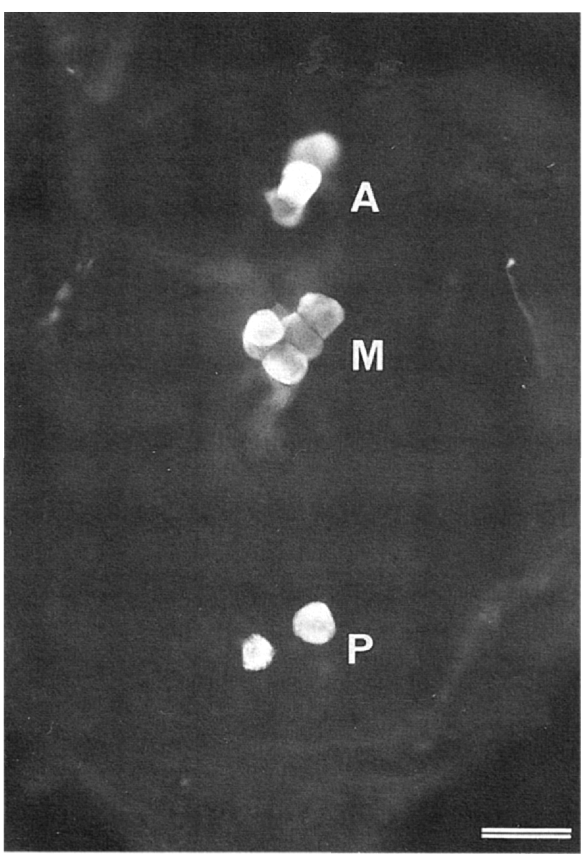

Fig. 1. Distribution of PBAN immunoreactive cells in the suboesophageal ganglion of a pharate pupa. Ventral view of a whole mount preparation. A: anterior cluster (4 cells); M: medial cluster (6 cells); P: posterior cluster (2 cells). Scale bar: 100 μm.

RESULTS

Figure 1 shows three clusters of DH/PBAN immunoreactive neurosecretory cells of a pharate pupa. Somata of the neurosecretory cells are located just under the neural sheath covering the ventral surface of the suboesophageal ganglion. The anterior, medial, and posterior clusters consist of four, six and two cells, respectively.

In the first experiment, the SG was only transected at the site between the medial and posterior cells or either half of the SG was removed. Females with a transected SG showed a strong DH activity comparable to that of the intact and shamoperated females. A strong DH activity was also observed in females with a posterior half of the SG whereas DH activity of females with an anterior half of SG did not differ from that of females without the SG (Fig. 2A). In contrast, a strong

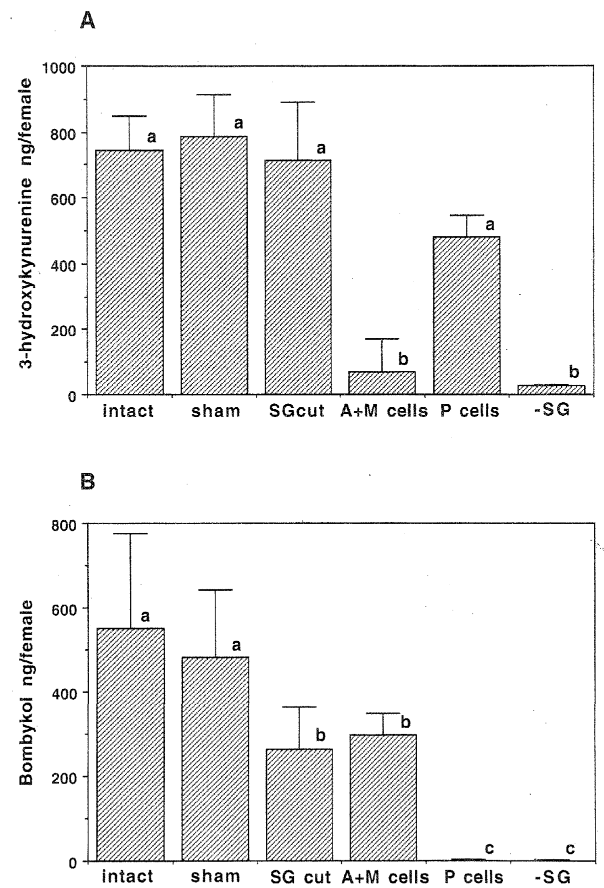

Fig. 2. Effects of complete or partial removal of the suboesophageal ganglion (SG) on 3-hydroxykynurenine content in the ovary (A) and on pheromone content in the pheromone gland (B). intact, intact females; sham, sham-operated females; SG cut, females in which the SG was transected into anterior and posterior halves; A+M cells, females that have an anterior half of SG containing the anterior and medial cell clusters; P cells, females having a posterior half of SG containing the posterior cell cluster; –SG, females lacking the SG. Each bar represents a mean of the content or the titer from 10 females with standard error of the mean. Different letters (a-c) indicate statistically different groups ($P \leq 0.05$, Mann-Whitney *U*-test).

pheromonotropic activity was obtained from females in which the anterior half of the SG was remained, though transection of SG itself did have an effect on pheromone production (Fig. 2B). Females with the posterior half of SG, like females lacking the SG, produced little pheromone.

In the second experiment, all or two clusters of somata of the DH/PBAN immunoreactive cells were carefully removed using a fine needle. The strongest DH activity was obtained from females retaining the posterior cells, though a substantial DH activity remained even when all somata was removed (Fig. 3A). A strong pheromonotropic activity was localized at the medial cells (Fig. 3B). Females retaining posterior cells produced only a small amount of pheromone, comparable to that of females lacking all somata.

When the SG was transected in the first experiment, the neurosecretory cells should be injured. The injury may be serious for the posterior cells that extend dendritic and axonal processes toward the anterior region of the SG (Ichikawa *et al.*, 1995). It is important to know how the injured cells regenerate their (axonal) processes for the secretion of neurohormones. Thus, complete structures of some posterior cells present in the transected SG were examined by injecting Lucifer Yellow. They appeared to sprout a few aberrant processes from the cut end of the dendrites and axons to form a meshwork of varicose processes around the surface of the SG and scar tissue formed over the lesion (Fig. 4).

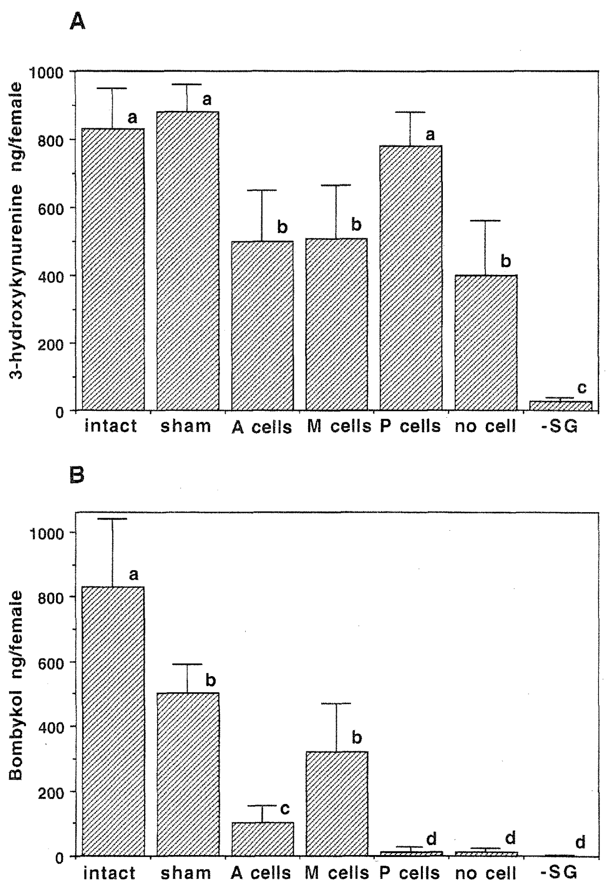

Fig. 3. Effects of complete or partial removal of the three clusters of DH/PBAN immunoreactive cells in the SG on 3-hydroxykynurenine content (A) and on the pheromone titer (B). intact, intact females; sham, sham-operated females; A cells, females in which somata of the medial and posterior clusters of the cells were surgically removed to retain the anterior cell cluster; M cells, females retaining only the medial cluster; P cells, females retaining only the posterior cluster; no cells, females with all somata removed. –SG females lacking the SG. Each bar represents a mean of the content or the titer from at least 7 females with standard error of the mean. Different letters (a-d) indicate statistically different groups ($P \leq 0.05$, Mann-Whitney *U*-test).

DISCUSSION

Fukuda and Takeuchi (1967) found a pair of somata of neurosecretory cells localized along the ventral midline of SG and suggested that these cells might be the source of a diapausing hormone (DH) because females laid non-diapause eggs when they had the cells surgically removed at the earliest pupal stage. The posterior cells examined in the present study may correspond to the neurosecretory cells because of the similarity in morphological and functional natures. Our observations suggest that the diapause induction activity is mainly assigned to posterior cells and the pheromonotropic activity relates to medial cells (Figs. 2, 3). However, this does not simply mean that the two classes of neurosecretory cells release particular single species of neuropeptide for such hormonal activities. DH, PBAN and three other neuropeptides (α-, β-, and γ-SGNP), all of which are generated from a common precursor polyprotein, often have considerable cross-activity (Sato *et al.*, 1993). Diapause induction activity may be possibly related to action of DH that may be released from the posterior cells, because PBAN and the three SGNPs exhibit weak or little DH activity. In contrast, the pheromonotropic activity is not attributable to (only) action of PBAN that may be released from medial neurosecretory cells, because β-SGNP shows potent pheromonotropic activity (Sato *et al.*, 1993). Although DH also has potent pheromonotropic activity, the hormone is unlikely to be a candidate for the functional pheromonotropic peptides in the female moth, because little DH activity was detected in extracts from the SG at the adult stage, a time when a large amount of pheromonotropic peptides should be produced in the SG for stimulating the biosynthesis of pheromone (Sonobe *et al.*, 1977).

In vertebrates, multiple bioactive neuropeptides are initially synthesized as large common precursor polyproteins that are subsequently processed to mature peptides with their own biological activities (Mizuno and Matsuo, 1994). Different cells or tissues have different neuropeptides derived from a precursor protein, through specific sets of processing proteases. For example, a precursor polyprotein is cleaved to a few peptides such as corticotropin (ACTH) and β-lipotropin in cells of the anterior lobe of the pituitary while it is processed to peptides such as α-melanocyte-stimulating hormone (α-MSH) and β-endorphin in cells of the intermediate lobe of the pituitary (Mizuno and Matsuo, 1994). It remains to be determined whether functional differentiation among the three

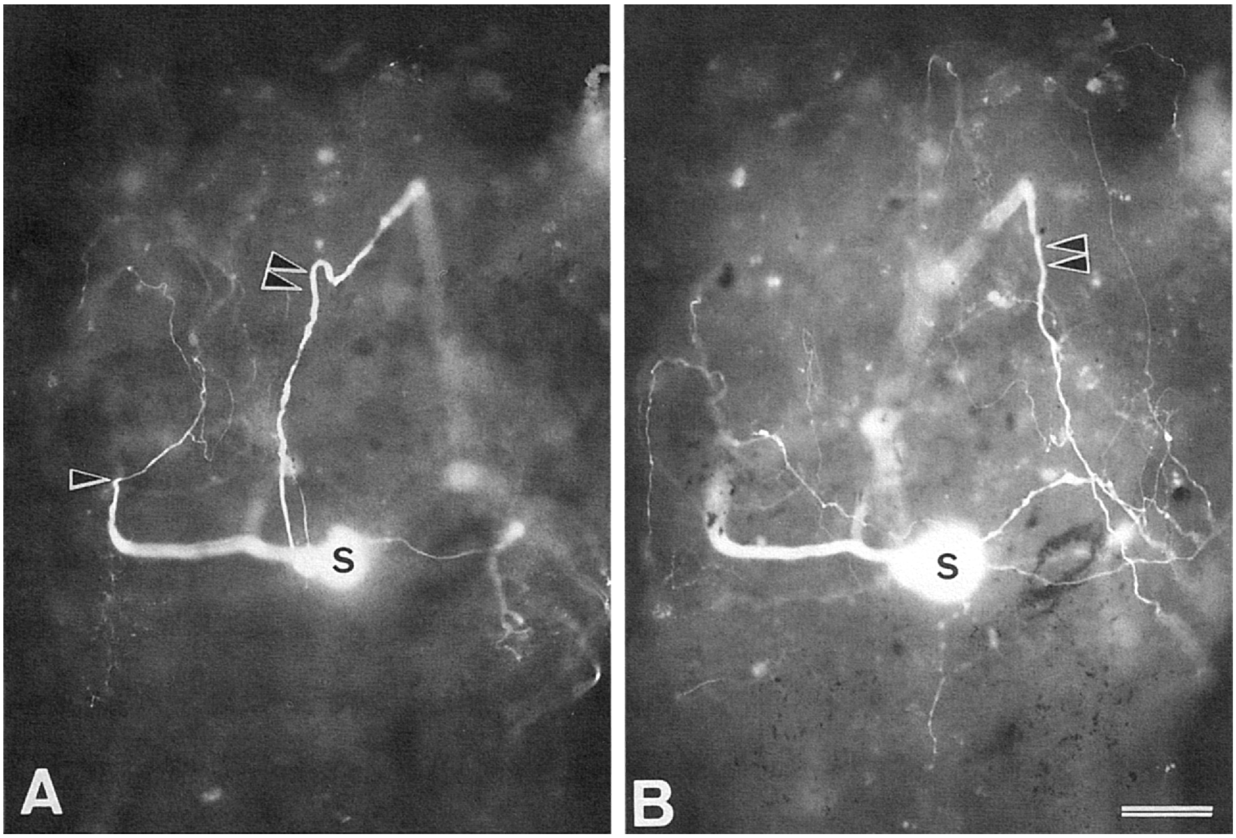

Fig. 4. Regeneration of processes of a posterior cells injured by transection of the suboesophageal ganglion. The cell was visualized by injecting Lucifer Yellow and then photographed at different focal planes (A, B). A meshwork of processes regenerated from cut ends of an axon (single arrowhead) and a dendrite (double arrow-heads) are formed on the surface of the ganglion (B). S, somata. Scale bar: 100 μm.

sets of DH/PBAN immunoreactive cells examined in the present study is due to different biochemical events. The antisera raised against N-terminal fragments of DH and PBAN may not be able to discriminate each of the three sets of neurosecretory cells (Fig. 1), because antibodies may bind to the precursor polyprotein and/or to its fragments.

DH acts on the developing ovary at the early and middle pupal stage while pheromonotropic peptides act on the pheromone gland after adult ecdysis. Thus, the posterior cells may be activated by a neural mechanism at the pupal stage while the medial cells may be activated by another neural mechanism at the adult stage. DH secretion is controlled by the brain (Fukuda, 1952; Matsutani and Sonobe, 1987; Morohoshi and Oshiki, 1969) and some GABAergic neurons may be involved in such a control mechanism (Hasegawa and Shimizu, 1990; Shimizu *et al.*, 1989). Because pheromone titers in virgin females change with a daily rhythm (Ando *et al.*, 1988), neurosecretory cells responsible for the pheromonotropic peptide secretion may be under the control of a circadian clock in the brain. Inactivation of pheromone production after mating suggests that an inhibitory neural mechanism is present in the central nervous system to suppress the activity of the neurosecretory cells (Ichikawa *et al.*, 1996).

It is difficult to exclude the possibility that medial and anterior cells play a minor role in DH and pheromonotropic activities, respectively (Fig. 3). We may be able to approach the problem by analyzing electrical activities of individual cells during pupal and adult stages. Such an approach is feasible as the three classes of DH/PBAN immunoreactive cells send an axon to the neurohaemal organ, the corpora cardiaca, via different neural pathways (Ichikawa *et al.*, 1995).

Surgical manipulations of the nervous system, such as a transection, extirpation and implantation of some nervous tissues have been employed in the studies of insect neuroendocrinology. However, little is known of the fate of neurosecretory cells after surgical manipulation. Insect neurons usually have highly-developed capacity of regeneration (Treherne *et al.*, 1988). Our present observations showed similar regenerative capacity of the neurosecretory cells of *Bombyx mori*. They produced several aberrant neurites that branch extensively to form a meshwork of varicose processes around the surface of SG and the scar tissue (Fig. 4). These varicose processes were also visualized immunocytochemically (data not shown), hence, they probably contain neurosecretory materials to be released into the haemolymph.

Insect neurons in the CNS often survive for more than several days even when separated from somata (Treherne *et al.*, 1988). We noted that some processes of the DH/PBAN immunoreactive cells remained in the SG even at the adult stage, though all somata of the cells had been surgically removed at the earliest pupal stage (data not shown). Females with all the somata of neurosecretory cells removed at the pupal stage have a substantial amount of 3-hydroxykynurenine in the ovary (Fig. 3A). The DH activity may be (in part) due to fractions of this diapause hormone that leak out from remnants of the cells during the pupal period.

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