Role of dopamine at the onset of pupal diapause in the cabbage armyworm *Mamestra brassicae*

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Received 11 June 1997; revised version received 24 June 1997

Abstract Experiments were conducted to examine the relationship between an onset of diapause and dopamine (DA) content in the cabbage armyworm *Mamestra brassicae* during pupation. The DA levels were significantly higher in haemolymph, integument and brain-central nervous system of diapause-destined pupae than in non-diapause-destined pupae. The elevated level of the integumental DA content was demonstrated to be due to an increase in dopa decarboxylase activity in diapausing pupal integuments through an enhancement of transcript levels of this enzyme. Elevation of the DA level accomplished by feeding L-DOPA to last instar larvae induced a diapause-like state in more than 50% of the pupae under long daylengths.

Key words: Dopamine; Insect; Pupal diapause; Growth-blocking peptide; L-DOPA; Brain-central nervous system

1. Introduction

Dopamine (DA) plays a fundamental role as a neurotransmitter in the mammalian central and peripheral nervous systems and is involved in a variety of important physiological and behavioral processes such as modulation of motor skills and higher-order cognitive function [1]. In the insect nervous system, DA is also the most abundant monoamine and it may serve as a neurotransmitter and neuromodulator [2,3]. Furthermore, in insects, extremely high concentration of DA is present in their integuments, where it serves as an essential intermediate of cross-linking agents in cuticle formation throughout insect development [4]. The two separate pools of DA, in the nervous tissues and integument, make it difficult to elucidate the functional role in endocrine physiology in insects.

Recent studies indicate that parasitization by parasitic wasps *Cotesia kariyai* elevates the level of a biogenic peptide, growth-blocking peptide (GBP), in the haemolymph of the host armyworm larvae *Pseudaletia separata* [5–7]. The elevated GBP disturbs the normal development of the host insect larvae by enhancing the DA level in haemolymph and nervous tissues [8]. Furthermore, during larval development, haemolymph GBP titer fluctuates synchronously with the DA level, and the GBP and DA peaks coincide with each molt period during which larvae temporarily cease moving and feeding [9,10]. This observation together with the fact that both GBP and DA have paralytic effects against lepidopteran larvae [9] allow us to speculate that a GBP-DA system might participate in the control of the induction of insect diapause.

The present study was conducted to examine this possibility by analyzing and perturbing the DA level in the cabbage armyworm *Mamestra brassicae* which has the capacity for diapause in the pupal stage under short daylengths [11,12].

2. Materials and methods

2.1. Animals

M. brassicae were reared on an artificial diet at 22°C in 16 h light:8 h dark (a long daylength) or at 18°C in 10 h light:14 h dark (a short daylength). More than 85% of larvae reared under long daylengths at 22°C were metamorphosed from pupae to adults by 2 weeks after onset of wandering stage. Therefore, those pupae were defined as non-diapause. Larvae reared under short daylengths at 18°C showed no signs of adult development by 7 weeks after onset of wandering stage. Those were regarded as diapause pupae. The cabbage armyworms maintained in this laboratory do not show a typical aestival-diapause by rearing under long daylengths at 22°C. Penultimate instar larvae undergoing ecdysis between 4 and 4.5 h after lights on were designated as Day 0 last instar larvae. The test larvae for L-DOPA treatment were reared at 22°C under long daylengths and fed an artificial diet containing 1% (w/w) L-DOPA during the last larval instar.

2.2. Chemicals

L-DOPA, pyridoxal phosphate and dopamine-HCl were obtained from Sigma. [α-^{32}P]dCTP was purchased from ICN, Biomedical Inc., USA.

2.3. Dopamine assay

A whole integument (approximately 1 cm width) between third and sixth segment and brain-central nervous system (Br-CNS) were dissected from the test pupa. Care was taken to remove adhering fat body from the both tissues. After washing with phosphate buffered saline (PBS, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2), the dissected tissues were lightly blotted with filter paper, weighed and immediately transferred to a 1.5 ml polypropylene microtest tube containing 50 μl ice-cold 0.2 N perchloric acid, homogenized in an ARTEK Sonic Dismembrator (20 pulses at 50 W) and centrifuged at 20 000×*g* for 10 min. A 25 μl aliquot of the resulting supernatant was directly injected onto the HPLC C_{18} extraction column (SHISEIDO Co., Japan, 4.6×150 mm, pore size = 120 Å). DA levels were determined by HPLC with coulometric electrochemical detection system (ESA 5100A, USA) [13].

The haemolymph sample (50 μl) was immediately transferred to a 1.5 ml polypropylene microtest tube containing 200 μl ice-cold 0.2 N perchloric acid and sonicated. The homogenate was centrifuged at 20 000×*g* for 10 min at 4°C. The supernatant was applied onto a 3 ml disposable C_{18} extraction column (J.T. Baker Inc., USA) after adjusting the pH to 9.1 with 1 N ammonium hydroxide. The fluid was drawn through the column using low vacuum pressure and then the column was rinsed sequentially with 10 ml water and 0.5 ml methanol. Biogenic amine was eluted from the extraction column with 1.5 ml methanol containing 10 mM trichloroacetic acid. The eluent was dried at 65°C under a stream of nitrogen and reconstituted with 40 μl of 0.2 N perchloric acid. A 20 μl aliquot was injected onto

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Abbreviations: Br-CNS, brain-central nervous system; DA, dopamine; DDC, dopa decarboxylase; GBP, growth-blocking peptide; L-DOPA, dihydroxyindoleacetic acid; PTTH, prothoracicotropic hormone

PII S0014-5793(97)00848-X

the HPLC column for the determination of DA as described previously [14].

2.4. Dopa decarboxylase (DDC) assay

Dissected tissues were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.2) containing 0.2 M sucrose and 0.1% phenylthiourea by sonication. The supernatant obtained after centrifugation at 10000×*g* for 1 min was assayed for DDC according to the method of Nagatsu et al. [15]. The reaction mixture (total volume: 50 μl) consisted of 0.1 M phosphate buffer (pH 7.2), 0.3 mM EDTA, 0.17 mM ascorbic acid, 0.01 mM pyridoxal phosphate, 0.5 mM L-DOPA, 0.1 mM pargyline hydrochloride and 5 μl DDC preparation. The mixture, without DDC preparation, was equilibrated at 25°C for 5 min and the reaction was started by adding the DDC preparation. Thirty minutes later, the reaction was terminated by adding 10 μl of ice-cold 3 M trichloroacetic acid. The reaction mixture was centrifuged at 20000×*g* for 10 min at 4°C and a 25 μl aliquot of the supernatant was directly injected into the HPLC C_{18} column to measure the DA level. DDC activity was calculated from the amount of DA formed enzymatically.

2.5. RNA isolation

Immediately after dissection of integument, it was transferred into liquid nitrogen and total RNA was isolated by using the method of Chomczynski and Sacchi [16]. Poly(A)$^+$ RNA was isolated from about 5 mg total RNA by using the Quickprep Micro mRNA Purification Kit (Pharmacia, Sweden).

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis

Oligonucleotide primers were designed from the consensus sequences reported for *Drosophila melanogaster* [17], *Aedes aegypti* [18] and *Manduca sexta* [19] DDC cDNA as follows: Primer 1, 5′-GG(CT)GT(GC)AC(AC)CACTGGCA-3′; and Primer 2, 5′-GG(AT)T(GC)(CT)TTCA(AG)CCACAT(AG)GC-3′. RT-PCR was performed with the Gene Amp XL RNA PCR kit (Perkin-Elmer, USA) basically according to the manufacturer's instructions. One hundred nanograms of total RNA isolated from *M. brassicae* integument was reverse-transcribed from oligo (dT) 16 primer. Forty cycles of the following PCR amplification with Primers 1 and 2 were performed using a temperature program 94°C for 1 min and 65°C for 3 min 45 s. The amplified 750-bp DNA was used as a hybridization probe, because this DNA fragment was hybridized with a cloned DDC cDNA of the armyworm *Pseudaletia separata*, a closely related species of the cabbage armyworm, under a high stringency.

Total RNA was size-fractionated on a 1.0% agarose gel after denaturation with glyoxal and dimethyl sulfoxide. Prehybridizations and hybridizations were done at 42°C (respectively for 2 and 12 h) in 50% (v/v) deionized formamide, 5×SSC, 50 mM phosphate buffer (pH 6.8), 2.5× Denhardt's solution, 0.1% (w/v) SDS and 0.1 mg/ml sheared salmon sperm DNA. After hybridization, the membrane was washed three times with 2×SSC containing 0.5% SDS at 50°C for 30 min according to the slightly modified protocol of Sambrook et al. [20]. The membrane was exposed to a BAS-1500 Imaging Plate (Fuji photo film Co., Japan) for 2 h at room temperature and quantitatively analyzed on an image analyzer BAS-1500.

3. Results

3.1. Dopamine level in haemolymph

The *Mamestra* larvae reared under short daylenghts at 18°C metamorphosed into diapause pupae 4 days after onset of wandering stage and all of them enter pupal diapause. However, the larvae maintained under long daylengths at 22°C developed into non-diapause pupae 3 days after onset of wandering stage (Fig. 1A).

The DA levels in haemolymph of prepupae and pupae reared under short and long daylengths were measured (Fig. 1A). Maximal DA levels were detected during the time of the

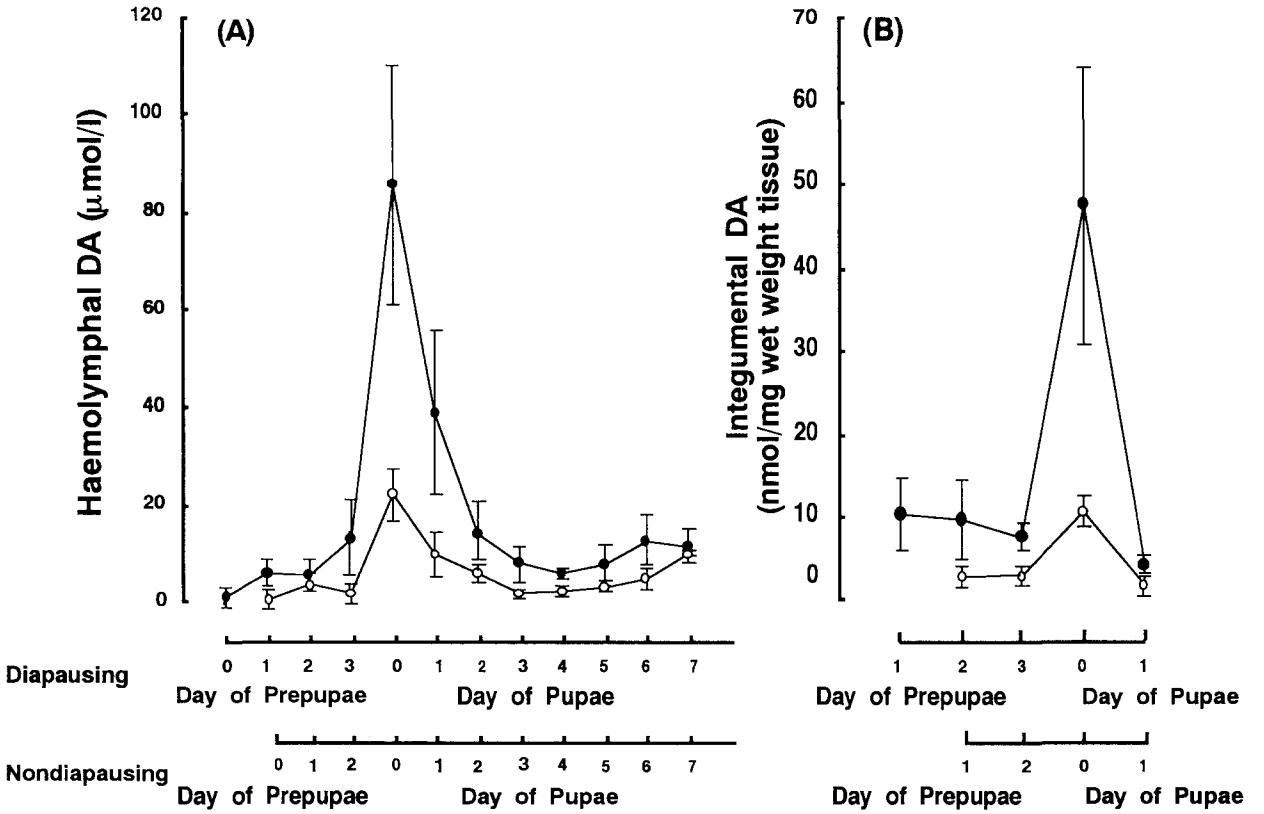

Fig. 1. Dopamine content in haemolymph (A) and integument (B) of diapausing and non-diapausing prepupae and pupae. Diapausing (●) and non-diapausing insects (○) were reared from first larval instar under short photoperiods and long photoperiods, respectively. DA contents are plotted as the means ± S.D. for 6 separate measurements.

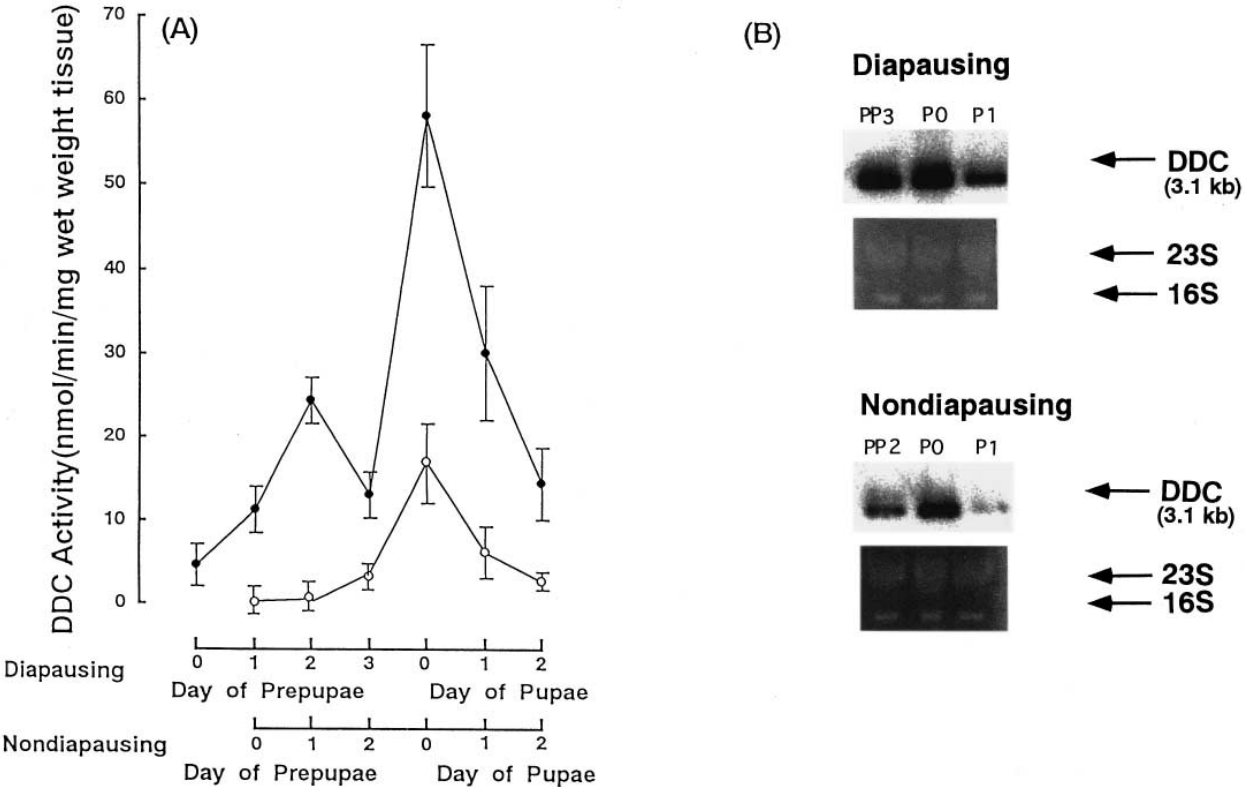

Fig. 2. Dopa decarboxylase activity (A) and its gene expression (B). (A) The enzyme activities are plotted as the means ± S.D. for 5 separate assays. (B) Northern blot (10 μg total RNA) probed with dopa decarboxylase cDNA fragment. PP, prepupae; P, pupae. Ethidium bromide staining of ribosomal RNA is shown under radioautogram. Other explanations as in Fig. 1.

molt from prepupae to pupae in both diapause-destined (diapausing) and non-diapause-destined (non-diapausing) pupae. The DA concentration in haemolymph of diapausing pupae was approximately 4 times higher than that in non-diapausing pupae at the molt period. Higher level of haemolymph DA in diapausing vs non-diapausing pupae were maintained throughout the prepupal and early pupal stages.

3.2. *Dopamine level and dopa decarboxylase activity in integument*

Because previous study [9] demonstrated that the predominant source of haemolymph DA is in the integument, the integumental DA levels in diapausing and non-diapausing insects were measured (Fig. 1B). The DA level in the integument was approximately 4 times higher in diapausing than in non-diapausing pupae at the molt period.

Based on previous reports [9,21], it was expected that the integumental DA is transformed from tyrosine absorbed from haemolymph through sequential catalysis by tyrosine hydroxylase and dopa decarboxylase (DDC) in epidermal cells of the integument. Preliminary studies showed that the integumental tyrosine hydroxylase activity in diapausing pupae was not significantly different from that in non-diapausing pupae (data not shown). However, in accordance with the difference of the integumental DA levels between diapausing and non-diapausing pupae, the integumental DDC activity was approximately 4 times higher in diapausing than in non-diapausing pupae (Fig. 2A). The results of Northern blot analysis showed that the relative abundance of the DDC gene expression was also much greater in diapausing than in non-diapausing pupal integument, thus indicating that the integumental DDC was transcriptionally enhanced during pupation in diapausing pupae (Fig. 2B).

3.3. *Dopamine levels in brain-central nervous system (Br-CNS)*

Because DA is one of the major biogenic amines in nervous tissues in insects, the DA contents in Br-CNS were measured in both diapausing and non-diapausing pupae (Fig. 3). Although a temporal increase was observed during pupation in non-diapausing pupal Br-CNS, the DA level was very low 1 day before and after pupation. In diapausing pupae, a large

Table 1
Induction of a diapause-like state by treatment with L-DOPA

Treatment	Total number of larvae	Number of pupae	
		Diapause-like	Non-diapause
Control	18	0	18
L-DOPA	18	10	8

All of control insects initiated adult development within 2 weeks after onset of wandering stage. Pupae which did not resume development by 7 weeks were defined as diapause-like pupae. Pupae not showing vermiculation by slightly touching were regarded as dead pupae. Six and two dead pupae were found in L-DOPA treated and control insects, respectively.

and rapid accumulation of DA was observed in Br-CNS, and the DA level was several times higher in diapausing than in non-diapausing insects during pupation.

3.4. Treatment with L-DOPA

To determine whether DA could participate in triggering pupal diapause in the *Mamestra* pupae, the last instar larvae reared under long daylengths at 22°C were treated with L-DOPA and observed their development. Preliminary studies were conducted to assess the change in the DA content in the pupae fed L-DOPA. By feeding last instar larvae of the cabbage armyworm with L-DOPA, the DA level was elevated by several times in all the tested tissues, haemolymph, integument and Br-CNS (Fig. 4). The treatment with L-DOPA resulted in developmental arrest in more than 50% of the tested pupae, although all control pupae metamorphosed into adults by 2 weeks after onset of wandering stage (Table 1). No indications of adult development such as eye pigmentation were observed in the development-arrested pupae by 7 weeks after onset of wandering stage.

4. Discussion

As demonstrated in many insects, the brains of diapausing pupae appear quite inactive. Key enzymes and metabolites that are abundant in an active brain during the period of intensive development, such as acetyl cholinesterase, cyclic nucleotides and octopamine, are only present in very small amounts [21–26]. Therefore, it is reasonable to expect that these factors control brain neurosecretory activity, although important regulatory sites are likely to be restricted to only a few neurosecretory cells; one of the lateral neurosecretory cells has been identified as the source of prothoracicotropic hormone (PTTH) by Agui et al. [27]. In non-diapausing insects the brain releases the PTTH required to initiate adult development shortly after the time of pupation. Decerebration prior to PTTH release locks pupae into a diapause-like state in which it will remain for many months [28]. Furthermore, it has been reported that removal of the prothoraric glands early in diapause traps the pupae permanently in diapause when

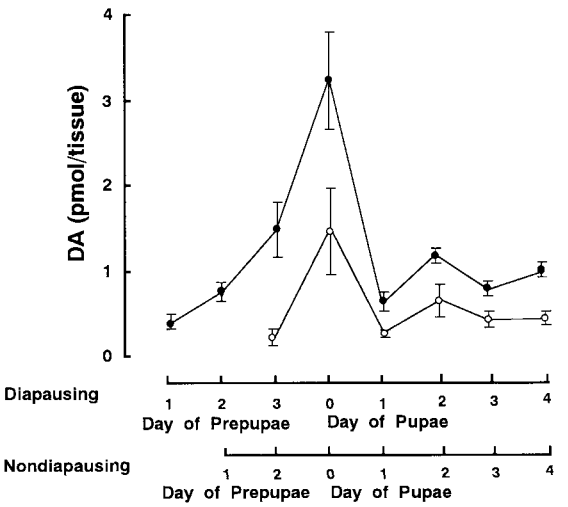

Fig. 3. Dopamine content in brain-central nervous system. DA contents are plotted as the means ± S.D. for 6 separate measurements. Other explanations as in Fig. 1.

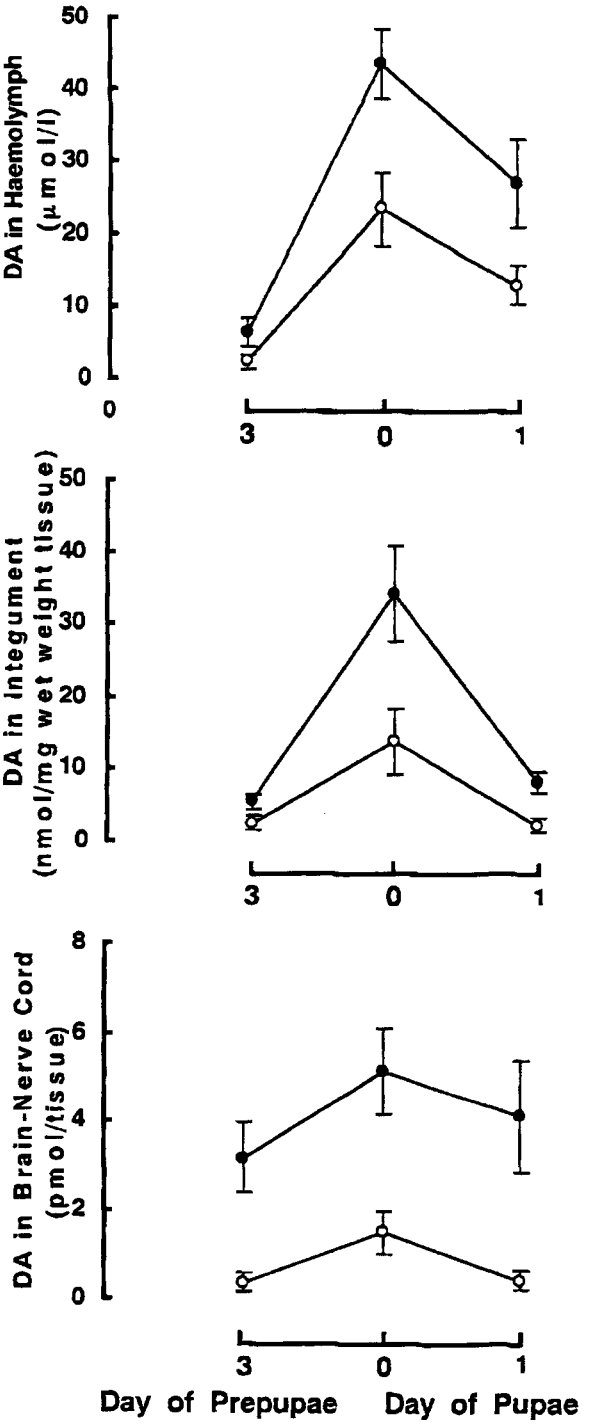

Fig. 4. Dopamine content in haemolymph, integument and brain-nerve cord of L-DOPA treated pupae. Test insects were reared under long daylengths and fed with an artificial diet containing 1% L-DOPA from last larval instar (●). Control insects were reared on an artificial diet under the same condition as the test insects (○). DA contents are plotted as the means ± S.D. for 5 separate measurements.

they were transferred to a favorable temperature [29]. Based on these results, it was thought that the brain would fail to exert its tropic action on the prothoracic gland by the release of PTTH in diapausing pupae. Endo et al. reported that PTTH was synthesized in diapausing pupal brains of the cabbage armyworm but it might not be secreted into haemo-

lymph [30]. However, there is no substantial evidence that a diapausing brain produces a factor that actively inhibits the secretion of PTTH.

The present study demonstrated that the DA content in Br-CNS is several times higher in a diapausing rather than in a non-diapausing cabbage armyworm during pupation. DA has been found to function as a neurotransmitter and to inhibit the release of the crustacean hyperglycaemic hormone from the eye stalk neuroendocrine complex of the red swamp crayfish, *Procambarm clarkii* [31]. Furthermore, it has been reported that DA antagonized the ovary-stimulating action of 5-HT in *P. clarkii* [32]. Using the information from these reports together with our present results showing the difference in the Br-CNS DA levels between diapausing and non-diapausing pupae, it is reasonable to expect that DA inhibits neurosecretion, such as the PTTH release, requisite for the initiation of adult development. This assumption was partly confirmed in the present study – which showed that the elevation of the DA level in the non-diapause-destined pupae, when treated with L-DOPA, induced a diapause-like state. However, the current study does not explain the mechanism by which DA prevents neurosecretion. Further analysis of the DA effects on PTTH release is required to substantiate this expectation.

The present study also indicated a higher level of DA in the haemolymph and integument of the diapausing pupae and L-DOPA treated pupae when compared to the non-diapausing pupae. As previously demonstrated [8,9], haemolymph GBP elevated by parasitization causes an increase in the haemolymph DA level through the stimulation of DA synthesis and secretion by the integument of the *P. separata* larvae. The observation that injection of DA itself or DA agonist, (−)-Quinpirole, disturbs the normal development of the armyworm larvae provides direct evidence that DA elevated in the haemolymph could cause the retardation of larval development [8]. It is undoubtedly important to evaluate the physiological role of haemolymph DA in insect development because insects have a large source of DA in the integument. At present, however, the mechanism whereby haemolymph DA exerts its inhibitory action on the nervous system which controls normal development remains unclear.

We could not characterize the contribution of GBP to the onset of pupal diapause because GBP has not yet been purified from the cabbage armyworm. The observation that GBP elevates the DA levels both in the integument [9] and Br-CNS (unpublished data) of the *P. separata* larvae indicates that GBP could contribute the onset of pupal diapause. Further investigations into the mechanism through armyworm may lead to a better understanding of the contribution of the GBP-DA system to insect neuroendocrinology.

Acknowledgements: We thank Dr. Kimio Shimada of this laboratory and Dr. Vladimir Kostal of Institute of Entomology, Academy of Sciences of the Czech Republic for their critical reading.

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