Stage-Dependent Changes of the Prothoracicotropic Hormone (PTTH) Activity of Brain Extracts and of the PTTH Sensitivity of the Prothoracic Glands in the Cabbage Armyworm, Mamestra brassicae, before and during Winter and Aestival Pupal Diapause

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ABSTRACT—The cabbage armyworm, *Mamestra brassicae*, enters winter and aestival diapause in the pupal stage under 10L-14D and 16L-8D, respectively, at 23°C. It develops without pupal dormancy under 14L-10D at 23°C. In 6th-instar larvae, the hemolymph ecdysteroid titers as well as the activity and sensitivity of the prothoracic glands (PGs) were nearly the same in the diapause- and nondiapause-programmed larvae. In non-diapause pupae (ND-pupae), the ecdysteroid titer peaked in the first half of the pupal stage. By contrast, in winter and aestival diapause pupae (WD- and AD-pupae), the ecdysteroid titer remained low after pupation. The ecdysteroid titer in diapausing AD-pupae was 8- to 10-times higher than that in diapausing WD-pupae. PGs from 5th-instar and 6th-instar larvae, as well as those from pupae, secreted ecdysteroids and showed a far stronger response to big prothoracicotropic hormone (PTTH) than to small PTTH *in vitro*. PG activity and its sensitivity to big PTTH fluctuated in a stage-dependent manner, and the pattern was nearly the same for 6th-instar larvae committed to develop into ND-, WD- or AD-pupae. When ecdysteroids for larval-pupal metamorphosis appears in the hemolymph of 6th-instar larvae, the activity and sensitivity of the PGs may be suppressed quickly. After pupation, the titer of hemolymph ecdysteroids as well as the activity and sensitivity of the PGs may be related to the strength (or duration) of pupal dormancy which differs between WD-pupae and AD-pupae.

INTRODUCTION

A cerebral neuropeptide, prothoracicotropic hormone (PTTH), activates ecdysteroid secretion by the prothoracic glands (PGs) (Williams, 1946; Ishizaki and Ichikawa, 1967; Chino *et al.*, 1974; Warren *et al.*, 1988) and is essential for growth and metamorphosis in insects (Wigglesworth, 1934; Fukuda, 1944).

The silkmoth, *Bombyx mori*, has two molecular forms of PTTHs (30 kDa and 5 kDa), tentatively named *Bom*-PTTH (22K-PTTH) and bombyxin, respectively (Ishizaki *et al.*, 1983; Nagasawa *et al.*, 1984; Kiriishi *et al.*, 1992). These big and small PTTHs were found in several lepidoptera including *Manduca sexta* (Bollenbacher *et al.*, 1984), *Samia cynthia ricini* (Yagi *et al.*, 1995), *Polygonia c-aureum* and *Papilio xuthus* (Endo *et al.*, 1990). The big PTTH appears to be a genuine PTTH since the PGs showed a far stronger response to the big PTTH than to the small PTTH *in vitro* in these insects, as was demonstrated in *B. mori* (Kiriishi *et al.*, 1992).

A number of multivoltine lepidoptera, such as *Manduca sexta* and *Papilio xuthus*, enter winter diapause in the pupal stage under short-day conditions (Müller, 1955; Danilevskii, 1961; Saunders, 1976; Beck, 1980). In these diapause pupae, the PTTH (or brain hormone) does not appear to be secreted by the brains, and the titer of hemolymph ecdysteroids remains low throughout diapause (Williams, 1946; McDaniel, 1979; Loeb, 1982; Bradfield and Denlinger, 1980).

The cabbage armyworm, *Mamestra brassicae*, produces winter-diapause pupae (WD-pupae) under 10L-14D and

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aestival-diapause pupae (AD-pupae) under 16L-8D, both at 23°C. All larvae reared under 14L-10D at 23°C develop into non-diapause pupae (ND-pupae) (Poitout and Buees, 1977; Masaki, 1980).

M. brassicae was shown to have big and small PTTHs (M.W. >15 kDa and 4.5 kDa), both of which activated the PGs of 2-day-old 6th-instar larvae in vitro (Endo *et al.*, 1990). We examined how the titer of hemolymph ecdysteroids fluctuates differently in *M. brassicae* 6th-instar larvae destined to develop with and without pupal dormancy. Also, we monitored the activity of the PG and its sensitivity to the big-PTTHs *in vitro* in three types of insects, and measured the PTTH activity of brain extracts from the three types of insects using an *in vitro* assay.

MATERIALS AND METHODS

Animals

M. brassicae was maintained in the laboratory at Yamaguchi University for more than 15 years. Larvae were kept in transparent plastic containers (12 × 18 × 6 cm^3) and were subjected to one of the following lighting conditions: 10-hr light and 14-hr dark (10L-14D, short-day conditions), 14L-10D (long-day conditions) or 16L-8D (typical long-day conditions) at 23°C. All the larvae reared under 14L-10D developed into ND-pupae and the majority (>98%) of larvae reared under16L-8D developed into AD-pupae at 23°C. The ND- and AD-pupae required 17-25 days and 30-60 days respectively to complete adult development at 25°C. All larvae raised from the egg stage under 10L-14D at 23°C developed into WD-pupae which showed no signs of adult development within 60 days after pupation at 25°C. Larvae destined to develop into ND-, WD- and AD-pupae under 14L-10D, 10L-14D and 16L-8D at 23°C are referred to as ND-, WD- and AD-larvae, respectively. Newly molted 6th-instar larvae were sorted from stock cultures every evening to obtain larvae of approximately the same developmental stages.

Larvae were fed on an artificial diet (Agui *et al.*, 1975), and adults were fed on a 10% sucrose solution.

Extraction of PTTHs

Brains dissected from 6th-instar larvae and pupae of different stages were stored at -85°C. A batch of 300 brains was homogenized in 2.5 ml of ice-cold acetone with a glass homogenizer, washed in 1.0 ml ice-cold 80% ethanol and extracted with 1.0 ml Grace's medium (GIBCO Lab., New York, USA). Brain extracts were heated for 3 min at 95°C, cooled rapidly and centrifuged. At each step, insoluble material was separated by centrifugation at 12,000 × g for 20-30 min at 4°C.

Separation of small and big PTTHs

Brain extracts were put into tubes with an ultrafilter (Ultrafree C3LGCOO, Millipore Tokyo) passing molecules smaller than 10 kDa and centrifuged at 3,000 × g for 4-5 hr at 4°C. The resulting filtrates were used as small-PTTH fractions, while the residues were washed with 1.0 ml Grace's medium and were used as big-PTTH fractions.

Assay of hemolymph ecdysteroids

Five microliters of hemolymph was collected from each of 10 insects at 12:00-14:00 (2 hr beyond the middle of the photophase). Ecdysteroids in each sample were extracted with 400 µl of methanol and the titer was quantified by radioimmunoassay (RIA) (Borst and O'Conner, 1972).

Assay of PTTH activity

The PTTH activity of brain extracts was quantified by the *in vitro* assay (Bollenbacher *et al.*, 1979) using the PGs from 2-day-old 6th-instar ND-larvae. Pairs of the PGs were obtained by dissection in saline (Wyatt, 1961) at 12:00-16:00 and washed in 50 µl of Grace's medium for 30-60 min. One of each PG pair was incubated in Grace's medium alone (50 µl) and the other was incubated in Grace's medium (50 µl) containing one of the extracts. After a 1- or 2-hr incubation, the PGs were removed and the amount of ecdysteroids secreted into the incubation medium was quantified by the RIA method. The activation ratio (Ar) representing PTTH activity of the brain extract was obtained by incubations of 5-6 gland pairs. The Ar-value shows the amount of ecdysteroids secreted by the experimental gland divided by that secreted by the contralateral gland (Bollenbacher *et al.*, 1979).

Quantification of sensitivity of the PGs

Pairs of PGs from ND-, WD- and AD-larvae of different stages were obtained by dissection in saline at 12:00-14:00 and washed in 50 µl Grace's medium for 30-60 min. They were incubated in Grace's medium with or without brain extracts of 0-day-old ND-pupae (one brain-equivalent/50 µl). The ecdysteroids secreted in each incubation medium for 2 hr was quantified by RIA, and the rate of ecdysteroids secretion was calculated. The Ar-value showing the sensitivity of the PGs to big PTTH *in vitro* was obtained as described above.

Radioimmunoassay (RIA) of ecdysteroids

The ecdysteroids in each sample were quantified by the RIA (Borst and O'Conner, 1972) with ecdysone (Sigma Chemical Co., St. Louis, USA) as a standard. Antiserum raised against 20-hydroxyecdysone (Rhoto Pharmaceutical Ltd., Osaka, Japan) was obtained from the Meguro Institute, Osaka, Japan. This antiserum exhibits reactivity with 20-hydroxyecdysone approximately 5-times stronger than that with ecdysone and 3-dehydroecdysone. Radioactive ecdysone, (23, 24-^{3}H)-ecdysone (2.96 GBq/mmol), was obtained from New England Nuclear, Boston, USA. The minimal amount of ecdysteroids quantified by the RIA method was 2.5 pg(ecdysone equivalents)/tube.

RESULTS

Stage-dependent fluctuations of the titers of hemolymph ecdysteroids in 6th-instar larvae and pupae

To examine stage-dependent fluctuations of the hemolymph ecdysteroid titer under different photoperiodic conditions, newly molted 6th-instar larvae were sorted from stock cultures reared under 14L-10D, 10L-14D or 16L-8D at 23°C. Daily hemolymph samples for RIA were collected from larvae and pupae from the first day of the 6th instar to the 7th or 12th day after pupation. Five microliters of hemolymph was collected from each of 10 insects at different stages. AD-pupae whose hemolymph was collected for RIA were kept at 25°C to determine whether they formed spots (a sign of adult development). If the AD-pupa formed spots within 20 days, the hemolymph sample was discarded.

Early in the 6th instar the hemolymph ecdysteroid titers were low (5-10 pg/µl) in ND-, WD- and AD-larvae. The titer began to increase 0-2 days before the larvae stopped feeding and peaked 2 days before pupation in all types of larvae. Thereafter, the titers decreased quickly as the larvae approached pupation (Fig. 1a, b, c).

The ecdysteroid titer of ND-pupae increased after pupation and peaked (4.1 ng/µl) 3 days later. The titer decreased thereafter, and remained low in the second half of the pupal stage (Fig. 1a). In WD- and AD-pupae, the titer decreased as pupation approached, and remained low before

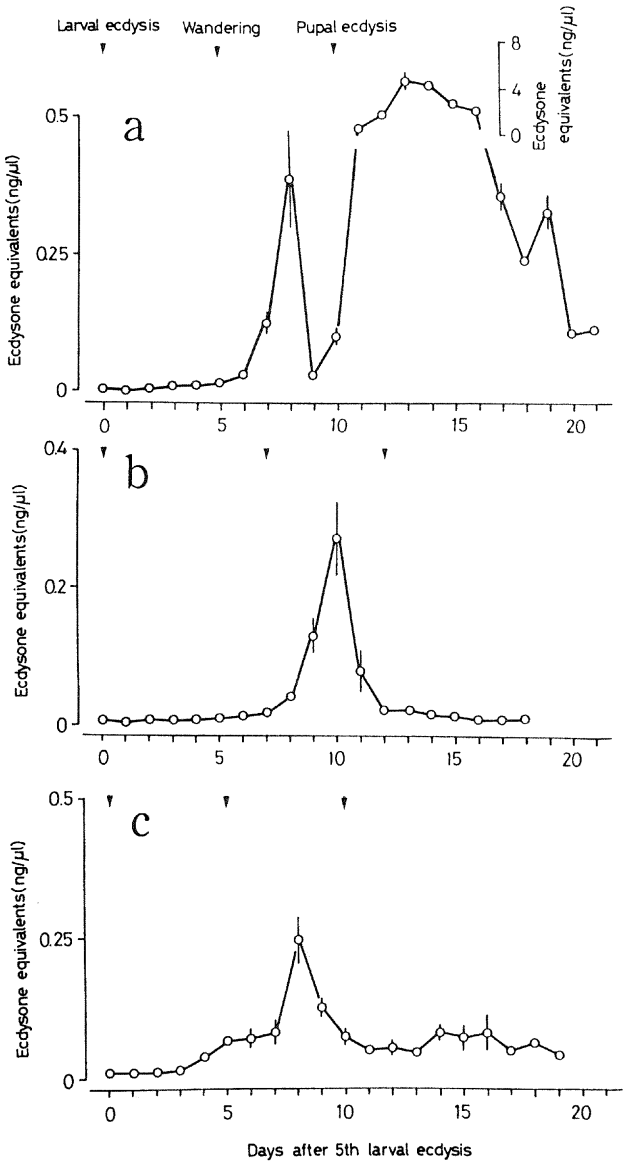

Fig. 1. Developmental changes of the titers of hemolymph ecdysteroids in larvae and pupae raised from the egg stage under 14L-10D (**a**), 10L-14D (**b**) and 16L-8D (**c**) at 23°C. Open circles and straight lines show the titers of hemolymph ecdysteroids and their standard errors, respectively. Solid arrows show the days of 5th larval ecdysis (left), wandering (middle) and pupation (right).

and during both winter and aestival diapause. The ecdysteroid titer in diapausing AD-pupae (40-80 pg/μl) was 8- to 10-times higher than that in diapausing WD-pupae (4-5 pg/μl) (Fig. 1b, c).

The number of days which larvae require for the 6th instar is different, but profiles of the fluctuations of the titers of hemolymph ecdysteroids do not vary in the ND-, WD- and AD-larvae of the 6th instar (Fig. 1a, b, c).

Responsiveness of the PGs to the big and small PTTHs in 5th-instar ND-larvae, 6th-instar ND-larvae and ND-pupae

To examine the responsiveness of PGs to big and small PTTHs, newly molted 5th- and 6th-instar larvae and newly molted pupae were sorted from a stock culture raised from the egg stage under 14L-10D (long-day conditions) at 23°C. Pairs of the PGs isolated from 2-day-old 5th-instar ND-larvae, 2-day-old 6th-instar ND-larvae and 2-day-old ND-pupae were incubated in Grace's medium with or without big- and small-PTTH fractions. The dosage of big- and small-PTTH fractions added to the incubation medium was changed from 1/8 to 4 brain-equivalents/50 μl.

The PGs isolated from 5th-instar and 6th-instar ND-larvae as well as those from ND-pupae showed a dose-dependent response to big-PTTH and small-PTTH fractions *in vitro* (Fig. 2). The doses of the big-PTTH fraction giving maximal Ar-values for the PGs of 5th-instar ND-larvae, 6th-instar ND-larvae and ND-pupae (Ar 10.0, Ar 8.0 and Ar 11.0) were 2.0, 2.0 and 1.0 brain-equivalents/50 μl, respectively. Half maximal Ar-values were obtained by doses of 1/2, 1/4 and 1/2 brain-equivalent/50 μl, respectively (Fig. 2).

The titers of small-PTTH fraction for inducing a detectable PG response (Ar > 2) were 2, 2 and 4 brain-equivalents/50 μl in the PGs of 5th-instar ND-larvae, 6th-instar ND-larvae and ND-pupae, respectively (Fig. 2).

The results indicate that responsiveness of the PGs to the big and small PTTHs does not vary among the young 5th-instar larval, the young 6th-instar larval and the young pupal stages. With respect to the induction of the minimal response of the PGs, the small-PTTH fraction requires 8- to 10-times larger doses than the dose of the big-PTTH fraction.

Stage-dependent fluctuations of activity of the PGs and their sensitivity to brain-extracts in three types of 6th-instar larvae and pupae

To investigate how the PG activity and the sensitivity of the PGs to big PTTH change during the 6th-instar larval and the early pupal stages, newly molted 6th-instar larvae and newly molted pupae were sorted from stock cultures raised from the egg stage under 14L-10D, 10L-14D or 16L-8D at 23°C. Pairs of PGs isolated from 6th-instar larvae and pupae were incubated in Grace's medium, one with and the other without the brain extract of 0-day-old ND-pupae (one brain-equivalent/50 μl). Daily collections of PGs were isolated from day 0 of the 6th instar to day 0 or 1 of pupae.

All PGs incubated in Grace's medium alone secreted ecdysteroids. The activity of the PGs, i.e. the rate of ecdysteroid secretion, varied with the stage of the PG donors (Fig. 3a, b, c). The PGs isolated from ND-, WD- and AD-larvae of the early 6th instar secreted ecdysteroids at a rate of 25-30 pg/gland/hr. Activity increased as the days passed and reached a peak (2.8-3.8 ng/gland/hr) on day 4-6 of the 6th instar (0-3 days before wandering). Thereafter, it decreased as pupation approached (Fig. 3a, b, c).

The activity of the PGs increased when they were treated with brain-extract (one brain-equivalent/50 μl) *in vitro*. The fluctuating patterns which the treated PGs showed with the developmental stages were similar to those in the contralateral PGs incubated in Grace's medium alone (Fig. 3a, b, c). The

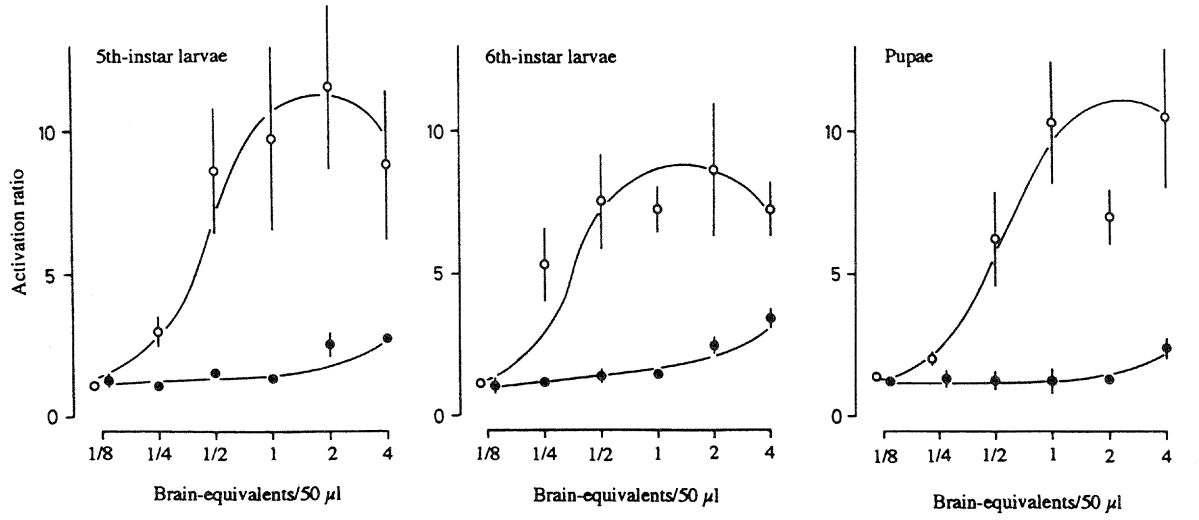

Fig. 2. Dose-dependent response of the PGs to the big and small PTTHs. PGs were obtained from 5th-instar larvae (left), 6th-instar larvae (middle) and pupae (right) raised from the egg stage under 14L-10D at 23°C. Open and solid circles show a response of the PG(s) (Ar-values) to big-PTTH and small-PTTH fractions with standard errors.

sensitivity of the PGs to the brain extracts of 0-day-old ND-pupae (one brain-equivalent/50 μl) was measured *in vitro*. Ar-values were 8.6, 11 and 1.9 in the PGs of ND-, WD- and AD-larvae respectively on the first day of the 6th instar. The Ar-values became larger as the days went by and reached a peak on day 2 (Ar 21) in ND-larvae and on day 4 (Ar 40 and Ar 8) in WD- and AD-larvae. Thereafter, the Ar-values decreased quickly and remained low and constant throughout the second half of the 6th-instar larval stage (Fig. 3a, b, c).

The activity of the PGs and their sensitivity to big PTTH were low on the first day of the 6th instar in all types of larvae. The activity and sensitivity of the PGs increased as the days went by and reached a peak 0-3 days and 2-3 days before wandering, respectively. Both activity and sensitivity appear to decrease quickly when a small amount of ecdysteroids appear in the hemolymph of the 6th-instar larvae.

Fluctuations of PTTH-activity in brain-extracts from ND-, WD- and AD-larvae

To investigate how the PTTH-activity of the brains fluctuates during the 6th-instar larval and pupal stages, brains were obtained from the three types of 6th-instar larvae and pupae at different stages. Batches of 600 brains were extracted with Grace's medium and PTTH activity was quantified by *in vitro* assay using PGs from 2-day-old 6th-instar ND-larvae.

The PTTH-activity in brain-extracts changed with the stages in ND-, WD- and AD-larvae of the 6th instar (Fig. 4a, b, c). PTTH-activity was low (Ar 5.4) on the first day of the 6th instar in ND-larvae and increased to a small peak (Ar 13.1) on day 3 (2 days before wandering). It decreased, thereafter, but increased again and reached a maximum (Ar 21.7) on day 7 (one day before the peak of the titer of hemolymph ecdysteroids) (Fig. 4a).

Fluctuations in the PTTH-activity of the brain seemed to be nearly the same among the three types of insects in the 6th-instar larval stage, but were clearly different in the pupal stage. In WD-pupae, the PTTH-activity increased rapidly after pupation (Fig. 4b), whereas in the AD- or ND-pupae, the activity did not increase or increased only gradually after pupation (Fig. 4c, a).

DISCUSSION

Pupae of *M. brassicae* are programmed to enter winter and aestival diapause by short and typical long days imposing during larval development. At intermediate daylengths, *M. brassicae* develops without pupal dormancy. The length of the stages from the 5th larval ecdysis to wandering and from wandering to pupation vary among ND-, WD- and AD-larvae, but the titers of hemolymph ecdysteroids is almost the same in all three types of insects (Fig. 1a, b, c). In the pupal stage, however, the ecdysteroid titers differ. In ND-pupae, the titer was low immediately after pupation, increases as the days passed and forms a large peak early in the pupal stage (Fig. 1a). The titers of hemolymph ecdysteroids of WD- and AD-pupae remained low after pupation (Fig. 1b, c), as is typical of other species that enter pupal diapause, e.g., *Hyalophora cecropia* (McDaniel, 1979), *Heliothis virescens* (Loeb, 1982) and *Manduca sexta* (Bowen *et al.*, 1985). Although the ecdysteroid titers remain low in both types of diapausing pupae, the titer in AD-pupae of *M. brassicae* was far higher than in WD-pupae (Fig. 1b, c).

The PGs of 5th-instar and 6th-instar ND-larvae, as well as those of ND-pupae, responded to the big and small PTTHs and increased secretion of ecdysteroids *in vitro* (Fig. 2).

Changes in PG activity and sensitivity to big PTTH were nearly the same in ND-, WD- and AD-larvae of the 6th instar (Fig. 3a, b, c). Activity and sensitivity of the PGs are increased

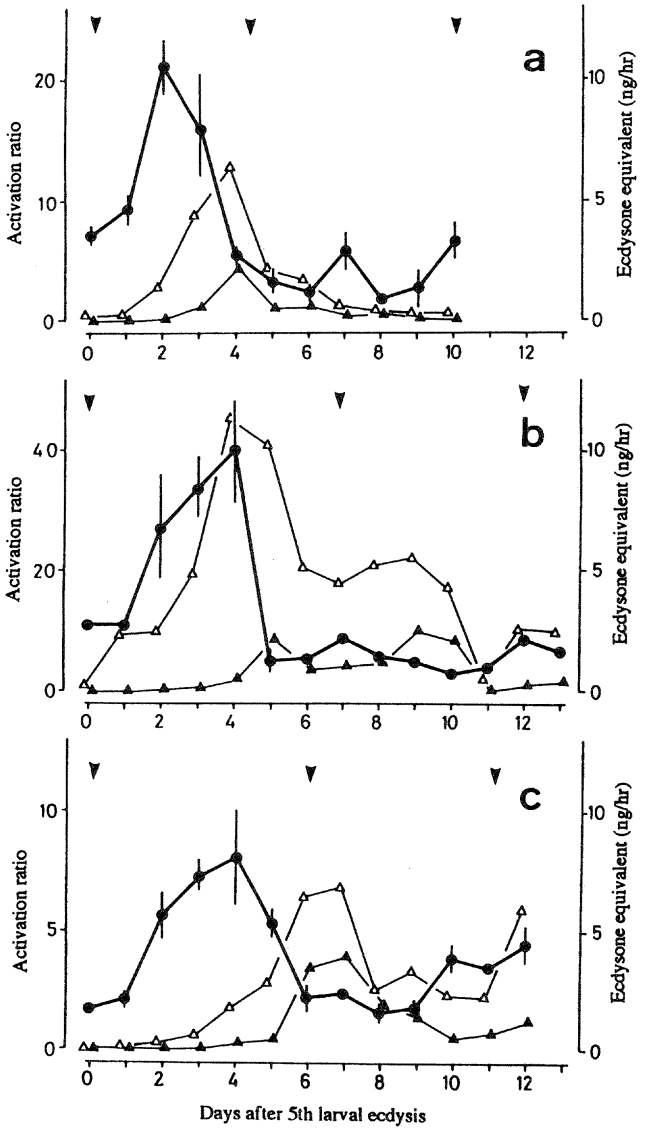

Fig. 3. Developmental changes of the PG activity and sensitivity of the PGs to a brain-extract in 6th-instar larvae reared under 14L-10D (**a**), 10L-14D (**b**) or 16L-8D (**c**) at 23°C. Open and solid triangles show the activities of the experimental and control PGs (the rate of ecdysteroid secretion), respectively. Solid circles show the responsiveness of the PGs (Ar-values) to the brain extract of 0-day-old ND-pupae (one brain-equivalent/50 μl). Solid arrows show the days of 5th larval ecdysis (left), wandering (middle) and pupation (right).

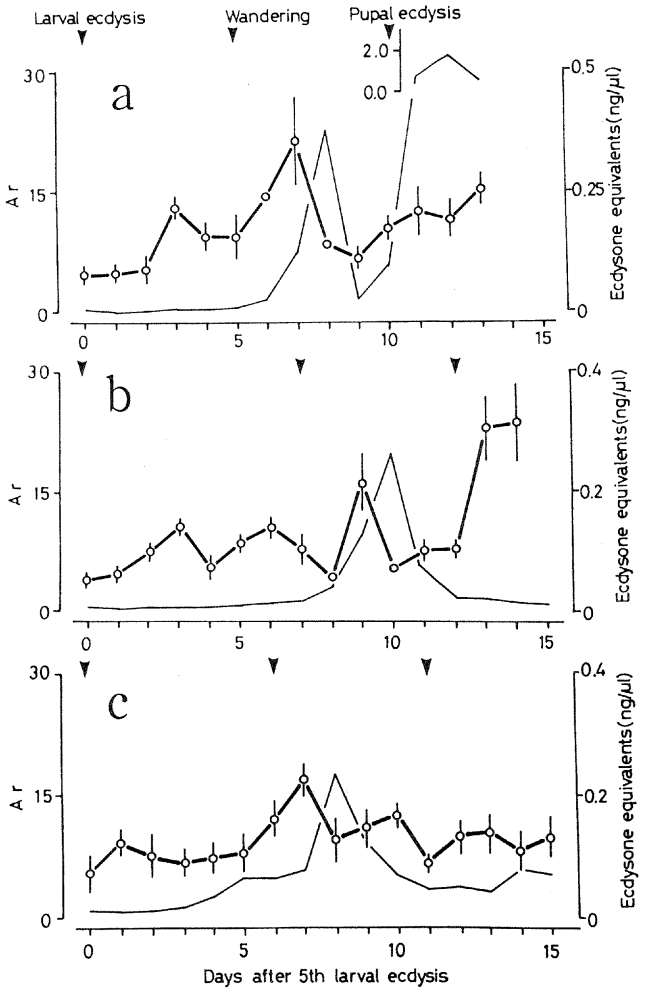

Fig. 4. Stage-dependent changes of PTTH activity in brain extracts of larvae and pupae raised from the egg stage under 14L-10D (**a**), 10L-14D (**b**) or 16L-8D (**c**) at 23°C. The PGs were obtained from 2-day-old 6th-instar ND-larvae. Open circles and straight lines show the PTTH activity of brain extracts (one brain-equivalent/50 μl) expressed in Ar-value and the standard errors. Thin straight lines show the fluctuations of the titers of hemolymph ecdysteroids, redrawn from Fig. 1 with some modifications. Solid arrows show the days of 5th larval ecdysis (left), wandering (middle) and pupation (right).

by big PTTH which is supposed to be secreted at low rates with a daily rhythm in the early 6th-instar larval stage as demonstrated in *B. mori* (Shirai *et al.*, 1995). They are peaked 2-3 and 0-3 days before wandering and suppressed quickly by ecdysteroids appearing in the larval hemolymph respectively (Fig. 3a, b, c).

For undergoing metamorphosis, the brains may synthesize and secrete a large amount of big PTTH which forces the PGs to secrete a large amount of ecdysteroids. The PTTH-activity of brain-extracts seems to fluctuate in an age dependent manner with the changes of the synthesis and secretion of big PTTH. The secretion of big PTTH for larval-pupal ecdysis may occur before the peak of hemolymph ecdysteroids. But, there remains an interesting fact that the activity of the PGs as well as their sensitivity to big PTTH decreased around the middle of the 6th-instar, when ecdysteroids appear in their hemolymph (Fig. 3a, b, c).

The titer of hemolymph ecdysteroids which we monitored

every day at 12:00-14:00 is close to the daily minimum in *M. brassicae* larvae. That is, the secretion of big PTTH begins daily at 11:00 and continues for about 8hr (Hasegawa *et al.*, unpublished). The titers of PTTH and ecdysteroids in the hemolymph may show a daily fluctuation (Agui and Hiruma, 1982; Shirai *et al.*, 1995) on a daily rhythm of PTTH secretion.

In ND-pupae, a large amount of big PTTH is secreted after pupation, but the secretion decreases within a few days. The big PTTH which is synthesized thereafter accumulates gradually as the days go by in the brains of ND-pupae (Fig. 4a). The brains of WD-pupae may synthesize but not secrete the big PTTH within a few days after pupation. The big PTTH may remain in their brains throughout winter diapause (Fig. 4b). In AD-pupae the brains may synthesize and secrete the big PTTH at a relatively low rate to maintain the PG activity and their sensitivity to the big PTTH before and during aestival diapause (Fig. 4c).

The role which a neuroendocrine system secreting the big PTTH plays in the photoperiodic regulation of the strength and duration of pupal dormancy may be revealed by from studying about these two types of diapause pupae of *M. brassicae*.

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