Cholinergic and Serotonergic Control of Prothoracicotrophic Hormone Release during the Pupal Stage of the Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)¹

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Prothoracicotrophic hormone (PTTH) is a neuropeptide hormone, which is synthesized in two pairs of dorsolateral neurosecretory cells in the brain of *Bombyx mori* and other insects, and released from the axonal ending in the corpus allatum (AGUI et al., 1980; MIZOGUCHI et al., 1990). The released PTTH then stimulates the prothoracic gland to synthesize and secrete ecdysone which elicits ecdysis (BOLLENBACHER et al., 1979; OKUDA et al., 1985). Consequently, PTTH is a trigger in the endocrine cascade regulating metamorphosis and morphogenesis in insects.

PTTH was first purified in the 1980's from *B. mori* (MATSUO et al., 1985; KATAOKA et al., 1987), and shown to be a homo-dimer with a molecular mass of about 30 kDa (KATAOKA et al., 1990). Subsequently, a cDNA encoding PTTH was cloned and the data deduced from the cDNA indicated that PTTH was synthesized as a 224-amino acid polypeptide pre-

cursor and that 109 amino acids in the carboxyl terminal region corresponded to a subunit of PTTH (KAWAKAMI et al., 1990).

Meanwhile, a radioimmunoassay (RIA) for ecdysone (BORST and O'CONNER, 1974) and an *in vitro* system to monitor PTTH titer in *Manduca sexta* have been successively developed (BOLLENBACHER et al., 1979; CARROW et al., 1981). These technical establishments enabled determination of the dynamics of PTTH titer in the hemolymph and examination of the role of neurotransmitters in the release of PTTH. We also developed a highly sensitive RIA for ecdysone using monoclonal antibody (SHIRAI et al., 1991) and an effective *in vitro* system to evaluate PTTH released from the brain-corpus cardiacum-corpus allatum (BR-CC-CA) of *B. mori* (SHIRAI et al., 1993). Using these systems, we clarified the timing of PTTH release into the hemolymph at the 5th-larval stadium (SHIRAI et al., 1993), and found that carbachol (acetylcholine agonist) and serotonin could induce the BR-CC-CAs of larvae on day 3 at the 5th stadium to release PTTH (SHIRAI et al., 1994, 1995).

However, there has been no attempt to investigate the mode of involvement of PTTH release in adult development, although the dynamics of ecdysteroid content in the hemolymph during the pupal stage has been reported (CALVES et al., 1976). Therefore, in this study, the timing of PTTH release into the hemolymph was clarified in association with ecdysteroid secretion in pupae of *B. mori*. Moreover, the question as to whether carbachol and serotonin induce PTTH release during the pupal stage was assessed.

MATERIALS AND METHODS

Collection of hemolymph specimens. All larvae of *B. mori* (Tokai × Fuyou) were reared on an artificial diet, KIT-32 (Nikko Shoji Inc.) at 25°C under dark conditions until the 3rd ecdysis, and under a 12 h light/12 h dark cycle through the 4th-, 5th-larval stadia and the pupal stage. The day of pupal ecdysis was designated as day 0 of the pupal stage. Hemolymph specimens were collected daily from the day before pupal ecdysis to day-6 pupa. All pupae used in the experiments were males.

Determination of ecdysteroid content. Ten microliters of

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hemolymph was collected from each of 5 pupae at the beginning of the photophase, from which ecdysteroid was extracted into 300 μ l of methanol. After samples were vortexed and centrifuged at $13,000\times g$ for 5 min, 100 μ l of the supernatant was subjected to RIA as described previously (SHIRAI et al., 1991). In this case, 20-hydroxyecdysone was used as the standard for the calibration curve.

Assay of PTTH titer. Approximately 5 ml of hemolymph were collected from 25 pupae including 5 pupae used for determination of ecdysteroid content, at the beginning of the photophase. Subsequently, PTTH was partially purified through the sequential steps of heat treatment (85°C, 5 min), and precipitations with ammonium sulfate (0.7 sat.) and cold acetone (55%, v/v), as described by MATSUO et al. (1985). The resulting PTTH preparation was subjected to an *in vitro* assay of PTTH titer, which included RIA for released ecdysone from cultured prothoracic glands with PTTH (SHIRAI et al., 1993). One unit of PTTH titer was defined as the minimal amount of the hormone which caused adult development in the debrained pupae (MATSUO et al., 1985).

Stimulation of BR-CC-CA with neurotransmitters. In GRACE's insect medium (Gibco Inc.), BR-CC-CAs were dissected out from 12 of 25 pupae used for hemolymph collection, at the beginning of the photophase of each day. They were mounted on a cellulose-acetate membrane filter (TOYO Roshi, ϕ 13 mm, 0.2 μ m) and fixed in a filter holder (Sartorius, 16514E). For washing out the trapped hemolymph, BR-CC-CAs were superfused with GRACE's insect medium for 30 min at a flow rate of 150 μ l/min using a Pharmacia p-500 pump (SHIRAI et al., 1995). Twelve prewashed BR-CC-CAs were separated into 3 sets, one of which consisted of 4 BR-CC-CAs. One set of these was incubated in 200 μ l of GRACE's insect medium containing 1 mM carbachol or 1 mM serotonin in a micro test tube (Eppendorf, 0.5 ml) for 1 h at 25°C under light conditions. As a control test, a set of BR-CC-CAs was incubated in GRACE's insect medium alone. The incubation medium was heated at 85°C for 5 min and centrifuged at $13,000\times g$ for 5 min. The supernatant was dialyzed against distilled water with an acetylated visking tube at 4°C for 3 days, and lyophilized. The resulting material was dissolved in 100 μ l of GRACE's insect medium and analyzed for PTTH titer.

Determination of serotonin in the brain. Ten brains dissected daily at the pupal stage were homogenized at 0°C in 50 μ l of 0.1 M perchloric acid by an ecno-

Fig. 1. Changes in PTTH titer and ecdysteroid content in the hemolymph during the pupal stage. Closed circles with vertical bars each indicate the mean and SE of PTTH titers in 3 independent determinations of PTTH titer by the *in vitro* assay. Closed squares indicate the mean of ecdysteroid contents of 5 individual determinations by RIA, which were represented as μ g of 20-hydroxyecdysone equivalent per ml of hemolymph.

Fig. 2. Developmental change in ability of the BR-CC-CA to release PTTH in response to carbachol and serotonin. Four BR-CC-CAs dissected out of pupae daily were incubated in 200 μ l of GRACE's insect medium containing 1 mM carbachol (A) and 1 mM serotonin (B). The PTTH titer in each medium was determined by *in vitro* assay. Each plot with a vertical bar indicates the mean and SE of 3 separate RIA for ecdysteroid released from cultured prothoracic gland with PTTH. Similar profiles were obtained for triplicate trials.

grind homogenizer (Radonoti Glass Technology Inc.), and then centrifuged at $13,000\times g$ for 10 min at 4°C . Analysis of serotonin in $20\ \mu\text{l}$ of the resulting supernatant was carried out twice by using an IRICA HPLC-ECD system, a high performance liquid chromatography system equipped with an electrochemical detector and a reversed-phase column (IRICA C₁₈: $4\times 150\ \text{mm}$). The mobile phase used was $0.05\ \text{M}$ sodium citrate (monobasic)– $0.075\ \text{M}$ sodium perchlorate monohydrate– $0.04\ \text{mM}$ EDTA disodium– 0.018% sodium-1-octane-sulphonic acid– 7.5% acetonitrile, pH 4.1, and the flow rate was kept at $0.6\ \text{ml/min}$. The detector potential was maintained routinely at $0.8\ \text{V}$ versus Ag/AgCl reference electrode.

RESULTS AND DISCUSSION

It has been reported that the ecdysteroid level in hemolymph at the pupal stage of *B. mori* changed with only one large peak over day 2 and no ecdysteroid was detected after day 6 until eclosion (CALVES, 1976), suggesting the possibility that PTTH enhancing the ecdysteroid level might be released into the hemolymph between day 0 and day 5 of the pupal stage. We then examined the following possibility. As shown in Fig. 1, ecdysteroid was first detected on day 1 and reached a maximal level (about $5\ \mu\text{g/ml}$) on day 2. A high ecdysteroid level lasted for another day and the level fell to the basal level on day 5. On one hand, the PTTH titer increased after the pupal ecdysis and reached a maximal level on day 2. Then, the titer decreased rapidly, and thereafter showed a small peak on day 4. The increase in PTTH titer accompanying secretion of ecdysteroid into the hemolymph implied that PTTH was released into the hemolymph between days 0 and 2. The PTTH titer in the small peak on day 4 might retard the lowering of ecdysteroid to a basal level. Figure 1, furthermore, showed that the maximal levels of PTTH titer (about 500 units/ml) and ecdysteroid content ($5\ \mu\text{g/ml}$) during pupal development were much higher in comparison with those in the 5th-larval stadium (SHIRAI, et al. 1993), being about 10 and 3 times higher, respectively. These rapid increases in the PTTH titer and the ecdysteroid content were essential for adult development.

Next, carbachol and serotonin were tested for their potency to induce the release of PTTH from the pupal BR-CC-CA. This experiment was prompted by the previous results that these neurotransmitters successively induced the release of PTTH from the BR-CC-CAs of day-3 larvae at the 5th stadium of *B. mori*

Fig. 3. Developmental change in serotonin level in the brain. Each plot indicates the mean amount of serotonin per brain for two analyses with an HPLC-ECD.

(SHIRAI et al., 1994). Figure 2 shows that carbachol was effective for the BR-CC-CAs of day-1 pupae and that serotonin was effective for the BR-CC-CAs of day-1 and day-4 pupae. PTTH released from the BR-CC-CAs of day-4 pupae was lower in titer than that from the BR-CC-CAs of day-1 pupae. In these experiments, the BR-CC-CAs of all ages released negligible amounts of PTTH in the control medium during the incubation period for 1 h (data not shown), resulting from the depression of spontaneous PTTH-release with a critical prewashing of BR-CC-CAs by the superfusion method, as described in a previous paper (SHIRAI et al., 1995). These results suggested that the acetylcholine- and the serotonin-modulated cascades were involved in PTTH release at the pupal stage, as well as at the larval stage (SHIRAI et al., 1994). The most striking aspect of the results, as shown in Fig. 2, was that the ability of BR-CC-CA to release PTTH in response to carbachol and serotonin changed developmentally. These changes in sensitivity of the BR-CC-CA to the neurotransmitters nearly coincided with the pattern of PTTH release into the hemolymph (Fig. 1). This suggested that the timing of PTTH release was controlled not only by neurotransmitter release but also by the sensitivities of BR-CC-CA to serotonin and carbachol, although the mechanisms of how the BR-CC-CA responded to serotonin and carbachol (via the specific receptors) remain to be elucidated.

The resemblance of the changing patterns in PTTH-release between Figs. 1 and 2B may reflect

the actual presence of serotonin in the pupal brain of *B. mori*. Therefore, developmental changes in serotonin content in the brain were determined. Figure 3 shows that the serotonin level in the brain fluctuated with two peaks on days 1 and 4. The maximal and basal levels of serotonin were about 230 pg and 50 pg per brain, respectively. It is noteworthy that the changes in serotonin level in the brain were consistent with those in sensitivity of the BR-CC-CA to serotonin (Fig. 2B) and with the fluctuation of the PTTH titer (Fig. 1). These results suggest that serotonin plays an important role in PTTH release *in vivo*.

The present results clearly showed that (i) PTTH release can be controlled with two different neurotransmitters, acetylcholine and serotonin; (ii) the BR-CC-CA released PTTH in response to these neurotransmitters at restricted periods of time during the pupal stage; and (iii) the level of serotonin in the brain increased when the BR-CC-CA was sensitive to serotonin for PTTH-release. These findings led us to conclude that, at the pupal stage of *B. mori*, serotonergic and cholinergic neurons are involved in the control of PTTH-release from the axon terminus of PTTH-neurosecretory cells in the BR-CC-CA system, thus contributing to adult development.

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