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CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN SILKWORM. DEVELOPMENTAL CHANGE OF CYCLIC AMP AND CYCLIC GMP PHOSPHODIESTERASES

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Summary

Changes in the activities of two cyclic AMP phosphodiesterases (II and III, EC 3.1.4.17) and cyclic GMP phosphodiesterase during development were studied in silkworm, *Bombyx mori*. The developmental patterns of the activities of the two cyclic AMP phosphodiesterases were similar to each other, but they differed distinctly from that of cyclic GMP phosphodiesterase. The ratios of cyclic GMP phosphodiesterase/cyclic AMP phosphodiesterase III were constant in larva, but changed greatly and rapidly before pupation.

Introduction

Recent observations have provided evidence that guanosine 3′:5′-monophosphate (cyclic GMP) plays as important a role in some mammalian cells or tissues as that ascribed to adenosine 3′:5′-monophosphate (cyclic AMP) and that it does not merely mimic the effect of cyclic AMP [1]. In insects, cyclic AMP has been suggested to be implicated in the action of some hormones [2—6]. Adenylate cyclase (EC 4.6.1.1) [4,7—9] and phosphodiesterase (EC 3.1.4.17) [10—15] have been identified, although so far there is no information available on the function of cyclic GMP and on the enzymes controlling the level of cyclic GMP. The level of cyclic nucleotides in any tissue not only depends on rates of synthesis by cyclase but on rates of hydrolysis by phosphodiesterase. It thus seemed of interest to study the developmental change of silkworm phosphodiesterase activity with regard to cyclic GMP as well as cyclic AMP.

In previous papers [14,15], we described the existence of multiple forms of cyclic nucleotide phosphodiesterase in silkworm; i.e., phosphodiesterase I which is a non-specific enzyme hydrolyzing both 3′:5′-cyclic nucleotides and 2′:3′-cyclic nucleotides, and two cyclic AMP-specific enzymes, phosphodiester-

ases II and III. These enzymes are different in their properties and can be separated from each other by column chromatography. This paper represents the developmental change of the activities of the two cyclic AMP phosphodiesterases and a cyclic GMP phosphodiesterase, which is probably a separate enzyme from the phosphodiesterase previously described in silkworm.

Experimental procedures

Materials

The eggs of silkworm (*Bombyx mori*) of a domestic strain, Gunka-Hohshun, were obtained from Gunze Co. Ltd, Kyoto. The worms were reared on mulberry leaves at 25°C. ^{3}H-labeled cyclic AMP (7.7 Ci/mmol) and cyclic GMP (10.2 Ci/mmol) were obtained from Daiichi Pure Chemicals Co., Tokyo, and purified by chromatography on a small column of aluminium oxide at pH 4.0 [16]. Other chemicals and reagents were as described previously [15].

Methods

Preparation of homogenate. Silkworm larvae from various developmental stages were homogenized in 5 vol. (v/w) of 0.01 M Tris/HCl, pH 7.5, containing 5 mM β-mercaptoethanol in a mortar and then in a motor-driven Teflon homogenizer at 0°C. The pupae were similarly homogenized except with the omission of homogenization in a mortar. The homogenate was centrifuged at 12 000 × *g* for 30 min at 2°C. The supernatant fluid was used for the assay of enzyme activities within 3 h after killing the animals. More than 90% of each activity of four phosphodiesterases present in the homogenate was recovered in the supernatant fraction.

Assay of cyclic nucleotide phosphodiesterases. The two-step assay for enzymatic activity was similar to that previously described [15]; 5′-[^{3}H]AMP or GMP formed by the phosphodiesterase was converted to the corresponding [^{3}H]nucleoside by the action of nuleotidase, unreacted substrate was removed by passing through a small column of aluminium oxide at pH 4.0 [16]. The [^{3}H]nucleoside was counted by liquid scintillation. The following reaction mixtures were used for separating the four phosphodiesterase activities. Reaction mixture A for the assay of phosphodiesterase I activity contained 50 mM Tris/HCl, pH 8.3, 5 mM $MgCl_2$, 4 mM cyclic [^{3}H]AMP and enzyme; Reaction mixture B for phosphodiesterase II contained 50 mM Tris/HCl, pH 7.8, 5 mM $MnCl_2$, 5 mM β-mercaptoethanol, 100 μg bovine serum albumin, 20 μM cyclic [^{3}H]AMP and enzyme; Reaction mixture B′ to assay the phosphodiesterase I activity contaminating the phosphodiesterase II assay was the same as Reaction mixture B except that 5 mM $MnCl_2$ was replaced by 10 mM EDTA; Reaction mixture C for phosphodiesterase III contained 50 mM Tris/HCl, pH 7.2, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 100 μg bovine serum albumin, 0.2 μM cyclic [^{3}H]AMP and enzyme; Reaction mixture D for cyclic GMP phosphodiesterase contained 50 mM Tris/HCl, pH 8.3, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 100 μg bovine serum albumin, 0.2 μM cyclic [^{3}H]GMP and enzyme. The reaction mixture in a total volume of 200 μl was incubated at 30°C for 15 min. The reaction was terminated by boiling for 90 s. After chilling, 20 μl of snake venom (5 mg per ml in 0.01 M Tris/HCl, pH 7.5) was added with an additional

incubation at 30°C for 15 min. After the addition of 50 μl of 0.8 M acetic acid, an aliquot was applied to a column (0.7 × 4.2 cm for cyclic AMP phosphodiesterase, 0.7 × 2.8 cm for cyclic GMP phosphodiesterase) of aluminium oxide equilibrated with 0.1 M ammonium acetate, pH 4.0, and eluted with the same buffer. The first 0.7 ml (for cyclic AMP phosphodiesterase) or 0.5 ml (for cyclic GMP phosphodiesterase) of eluate was discarded and the next 1.5 ml which contained more than 99% of adenosine or guanosine applied to the column was collected in a scintillation vial. When a possible metabolic derivative of cyclic [^{3}H]nucleotides, such as adenine, xanthine, hypoxanthine, inosine or guanine was applied to the column, more than 90% of each compound was eluted in the same fraction as adenosine or guanosine.

Specific activity was expressed as nmol substrate hydrolyzed per 15 min at 30°C per mg protein. Protein was measured by the method of Lowry et al. [17] using crystalline bovine serum albumin as standard.

Results

Separate assay of multiple phosphodiesterase activities

In silkworm larval extract, at least four enzyme activities concerning the hydrolysis of cyclic nucleotides have been observed; i.e., a non-specific enzyme which hydrolyzes both 2′:3′- and 3′:5′-cyclic nucleotides (phosphodiesterase I) [14], two cyclic AMP phosphodiesterases (phosphodiesterase II and III) [15] and a cyclic GMP phosphodiesterase (Morishima, I., unpublished data). These four enzyme activities could be separated from each other by column chromatography on DEAE-cellulose, Sephadex or hydroxyapatite and had some different properties in substrate specificity, K_m value, optimum pH and dependence on metal ions as summarized in Table I. Based on the differences in the properties, therefore, each of these phosphodiesterase activities could be assayed separately even in the homogenate under certain limited conditions as described in Methods. To demonstrate the separate assay of the phosphodiesterases in silkworm, the activities were assayed in various reaction mixtures using the homogenate and the partially purified phosphodiesterase preparations which were almost free from the contamination by the other phosphodiester-

TABLE I

PROPERTIES OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN SILKWORM

	Phosphodiesterase			
	I [14]	II [15]	III [15]	Cyclic GMP*
Substrate	3′ : 5′-cyclic purine nucleotides 2′ : 3′-cyclic nucleotides	Cyclic AMP	Cyclic AMP	Cyclic GMP
K_m	1.3 mM (cyclic AMP)	12 μM	0.2 μM	2 μM
Optimum pH	8.2 (cyclic AMP)	7.8	7.2	8.3
Requirement for metal ion	None	Mn^{2+}	Mg^{2+} or Mn^{2+}	Mg^{2+} or Mn^{2+}
Approx. mol. wt	66000	50000	97000	260000

* Morishima, I., unpublished data.

TABLE II

ACTIVITIES OF FOUR FORMS OF PHOSPHODIESTERASE ASSAYED IN VARIOUS REACTION MIXTURES

The activities were assayed in Reaction mixtures A, B, B′, C and D, which were as described in Methods and were used usually to assay the activities of phosphodiesterase I, phosphodiesterase II, phosphodiesterase I contaminating phosphodiesterase II, phosphodiesterase III and cyclic GMP phosphodiesterase, respectively. The enzyme preparations used were as follows: homogenate, prepared from fifth instar larvae of one day after the fourth ecdysis; phosphodiesterase I, partially purified by DEAE-cellulose column chromatography (peak I fraction in Fig. 2 of ref. 15); phosphodiesterase II, partially purified by DEAE-cellulose and Sephadex G-100 and concentrated (peak II fraction in Fig. 3 of ref. 15); phosphodiesterase III, partially purified by hydroxyapatite and dialyzed (Morishima, I., unpublished); cyclic GMP phosphodiesterase, partially purified by hydroxyapatite and Sephadex G-200 (Morishima, I., unpublished).

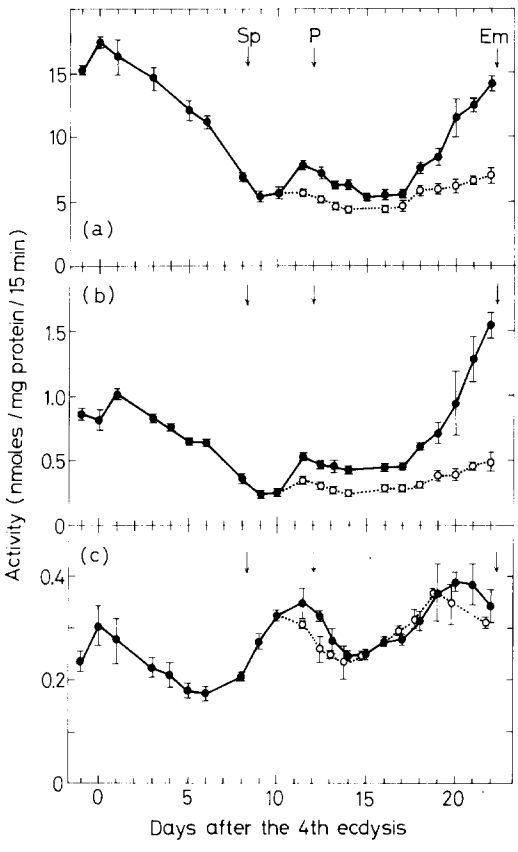
Enzyme	pmol hydrolyzed in reaction mixture					
	A	B	B′	(B-B′)	C	D
Homogenate (10 μl)	13500	970	43	927	58.0*	15.8
Phosphodiesterase I (10 μl)	11100	36	34	2	0.7	1.1
Phosphodiesterase II (10 μl)	640	621	1	620	1.2	0
Phosphodiesterase III (10 μl)	0	18	—	—	36.5*	0.6
Cyclic GMP phosphodiesterase (20 μl)	0	0	—	—	1.4	13.7
Homogenate (10 μl) + phosphodiesterase I (10 μl)	25100	997	80	917	58.2*	17.0
Homogenate (10 μl) + phosphodiesterase II (10 μl)	13800	1570	43	1527	58.6*	15.7
Homogenate (10 μl) + phosphodiesterase III (10 μl)	13400	992	—	—	95.1*	16.8

* The amount of enzyme used for the assay was reduced to 1/5 and the value was multiplied by 5 times.

ase. As shown in Table II, none of the partially purified phosphodiesterase had significant effect on the assay of any other phosphodiesterase activity. For example, an amount of phosphodiesterase II, which hydrolyzed 621 pmol of cyclic AMP in Reaction mixture B, hydrolyzed 640 pmol of cyclic AMP in Reaction mixture A. The latter value, however, corresponded to only a few percent of that usually observed when the phosphodiesterase I activity in the homogenate was assayed in Reaction mixture A. When the partially purified phosphodiesterase was added to the homogenate, similar results were obtained and the activities were additive. In some cases, however, contamination by phosphodiesterase I activity was not negligible in the assay of phosphodiesterase II activity. As phosphodiesterase I activity was not affected by the addition of EDTA [14] while phosphodiesterase II activity was completely inhibited by the compound [15], the activity of phosphodiesterase I was also assayed in Reaction mixture B′ which contained 10 mM EDTA instead of Mn^{2+} and then subtracted from that assayed in Reaction mixture B to give phosphodiesterase II activity.

Levels of phosphodiesterase activities during development

Changes in the activities of two cyclic AMP phosphodiesterases (II and III) and cyclic GMP phosphodiesterase during development were examined in the whole homogenate of silkworm. Fig. 1a, b and c shows the changes in the activities of phosphodiesterases II and III and cyclic GMP phosphodiesterase, respectively, from one day before the fourth larval ecdysis to the period of emergence of the moth. During this period the activities of phosphodiesterases



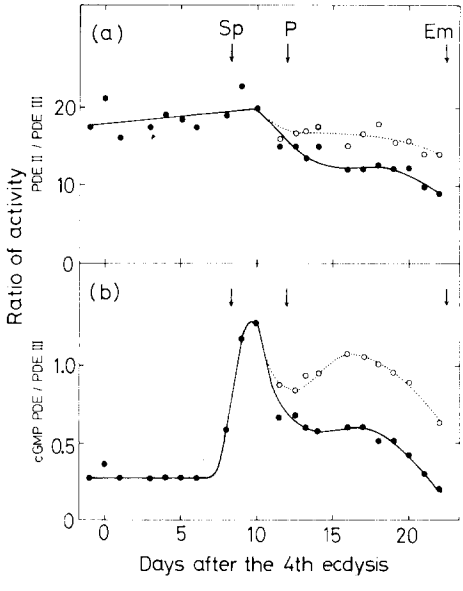


Fig. 1. Changes in the activities of cyclic nucleotide phosphodiesterases during silkworm development. Each value represents the mean of three experiments in duplicate assay; brackets indicate S.E. Sp P and Em represent the periods of start of spinning, pupation and emergence, respectively. a, cyclic AMP phosphodiesterase II; b, cyclic AMP phosphodiesterase III; c, cyclic GMP phosphodiesterase. ●———●, male; ○- - - - -○, female.

Fig. 2. Ratio of activities of cyclic nucleotide phosphodiesterases (PDE). Data were calculated from Fig. 1. a, cyclic AMP phosphodiesterase II activity/cyclic AMP phosphodiesterase III activity; b, cyclic GMP phosphodiesterase activity/cyclic AMP phosphodiesterase III activity. ●———●, male; ○- - - - -○, female.

II and III showed a similar developmental pattern; after the fourth larval ecdysis both activities were gradually decreased, and relatively lower levels were maintained during pharate pupal and pupal stages. In the male, however, the activities greatly increased with pharate adult development, while in the female, no remarkable increase was observed. The level of activities in female was lower than that in male throughout the periods of pupa and pharate adult, though no difference between both sexes was observed during the larval stage.

The ratios of the activity of phosphodiesterase II to that of phosphodiesterase III were calculated from Fig. 1a and b, and the changes in the ratio during the development are shown in Fig. 2a. The ratios of both activities were maintained constant throughout the larval stage, then gradually decreased with pupation and were maintained again almost constant during the pharate adult stage, though the level was somewhat lower than that of the larval stage.

The developmental pattern of cyclic GMP phosphodiesterase activity was greatly different from that of cyclic AMP phosphodiesterases (Fig. 1c). Three peaks of cyclic GMP phosphodiesterase activity were observed; at the period of the fourth ecdysis, one day before the pupation and two to three days before the emergence. The activity during pupa and pharate adult stages was relatively higher than that in the larval stage, in contrast to the lower activity of cyclic AMP phosphodiesterases in these periods. No remarkable difference was observed between male and female in the cyclic GMP phosphodiesterase activity in contrast to the cyclic AMP phosphodiesterase activities. It seems to be noteworthy that the increase in cyclic GMP phosphodiesterase activity occurred prior to the increases in the activities of cyclic AMP phosphodiesterases.

The ratios of the activity of cyclic GMP phosphodiesterase to that of cyclic AMP phosphodiesterase III were calculated from Fig. 1b and c, and are shown in Fig. 2b. A constant ratio was maintained in the larva. With the beginning of spinning, however, the cyclic GMP phosphodiesterase activity increased greatly compared to phosphodiesterase III activity and then rapidly decreased with the termination of spinning. In male pupa the ratio was maintained at a level two-times higher than that in larva, and the ratio decreased again with pharate adult development. In the female, about a two-fold higher level of the ratio was observed than that in the male.

Discussion

In the present study, it is demonstrated that the activities of multiple forms of cyclic nucleotide phosphodiesterase in silkworm can be separately assayed, even in homogenate, by selecting the assay conditions. Under the definitive assay conditions, the changes in the activities of three forms of cyclic nucleotide phosphodiesterase were studied with silkworm development. In these experiments, the phosphodiesterase II activity was assayed at 20 μM of cyclic AMP as substrate, which was presumably one to two orders higher than physiological concentration, and the phosphodiesterase III and cyclic GMP phosphodiesterase activities were assayed at presumably physiological concentration (0.2 μM of cyclic AMP or cyclic GMP, respectively).

The results show that the developmental patterns of the activities of the two cyclic AMP phosphodiesterases are similar to each other. The patterns are also similar to that of phosphodiesterase I described earlier [13]. Despite the great changes of the activities during development, the ratios of phosphodiesterase II to phosphodiesterase III are almost constant in the larval stage and in the pupal and pharate adult stages, though a slight decrease is observed at the period of pupation. This result suggests the possibility that the activities of the two cyclic AMP phosphodiesterases may be regulated not independently but by a common or closely related mechanism. In contrast, the developmental pattern of cyclic GMP phosphodiesterase is very different from that of cyclic AMP phosphodiesterases. The ratios of cyclic GMP phosphodiesterase to cyclic AMP phosphodiesterase III are maintained constant during larva, but the ratios increase greatly with the beginning of spinning, then rapidly decrease with its termination. The period of spinning, the stage of so-called pharate pupa, is the period in which the larval tissues are destroyed and reconstituted. The great

change in the ratios of the two activities suggest that the cyclic AMP and cyclic GMP phosphodiesterase activities are separately regulated. If the phosphodiesterase activities are under direct genetic control, the results could suggest that the two activities may be under separate genetic control. The separate genetic control of the two activities has been recently demonstrated in chicken embryonic fibroblasts [18].

Rudland et al. [19] recently showed that cultured mouse fibroblasts exhibit large differences in the concentrations of cyclic AMP and cyclic GMP and in the cyclic AMP/cyclic GMP ratios for growing and quiescent cells. It has also been proposed that growth of the mammalian cell is regulated by the balance of cyclic AMP and cyclic GMP in the cell [20]. Considering these observations and the "dualism" theory [1] of biological control through opposing action of cyclic AMP and cyclic GMP, the great changes of the cyclic AMP and cyclic GMP phosphodiesterase activities and of the ratios of the two activities during silkworm development are of great interest. However, the biological role of the multiple phosphodiesterases and the regulation mechanism of the activities are unknown. Furthermore, no information is available on the function of cyclic GMP in insect, although relatively higher levels of cyclic GMP in insect tissues have been reported [21—23]. At the present time, therefore, a clear explanation for the biological significance of the changes in the activities and in the ratios of the two activities cannot be given.

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