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CYCLIC NUCLEOTIDE PHOSPHODIESTERASES OF *HYALOPHORA CECROPIA* SILKMOTH FAT BODY

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Summary

Two soluble forms of 3′:5′-cyclic-nucleotide phosphodiesterase (3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) were found in the larval fat body of the silkmoth *Hyalophora cecropia*. These differ in elution profile on Sephadex G-200, solubility in ammonium sulfate, metal ion requirements and kinetic properties. Phosphodiesterase I has K_m values of 11 μM and 1.8 μM for cyclic AMP and cyclic GMP, respectively, has 5-fold greater maximal activity with cyclic AMP than with cyclic GMP, and is activated by Mg^{2+} and Co^{2+}, and inhibited by EDTA. Phosphodiesterase II has K_m values of 625 μM and 125 μM for cyclic AMP and cyclic GMP, respectively, has similar maximal activity with both substrates, and is not activated by divalent metal ions or inhibited by EDTA. Cyclic nucleotides and methylxanthines competitively inhibit both enzymes. Phosphodiesterase is found in both soluble and particulate fractions of homogenates. Total activity is highest during the larval stage of the insect, drops markedly following pupation, and rises again during pharate adult development.

Introduction

The regulatory roles of adenosine-3′,5′-cyclic monophosphate (cyclic AMP) in vertebrate tissues, particularly in relation to carbohydrate and lipid metabolism have received much attention [1]. Guanosine-3′,5′-cyclic monophosphate

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(cyclic GMP), which is usually present at much lower levels than cyclic AMP, appears to have regulatory activity distinct from, and sometimes antagonistic to the actions of cyclic AMP [2]. While understanding of the roles of cyclic nucleotides in insect tissues is still rudimentary, some evidence indicates a quantitatively greater role for cyclic GMP in insects than in vertebrate tissues. Some insect tissues, including silkmoth pupal fat body, have intracellular levels of cyclic GMP higher, relative to cyclic AMP, than those in most vertebrate tissues [3—6]. Both cyclic GMP- and cyclic AMP-dependent protein kinases have been detected in insect tissues, with particularly high activities of the cyclic GMP-dependent enzyme in silkmoth fat body [7,8]. Distinct cyclases catalysing the synthesis of these two nucleotides have been characterised from this same tissue with relatively high activities of guanyl cyclase [9]. Since the intracellular levels of cyclic nucleotides are determined by the rates of synthesis and degradation, both must be examined to understand the regulatory mechanisms.

Extracts of larval silkmoth fat body contain 3′:5′-cyclic nucleotide phosphodiesterase (3′-5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) activity which exhibits anomalous kinetics for both cyclic AMP and cyclic GMP [10]. Similar kinetics have been observed for phosphodiesterases from a wide variety of tissues, and are due at least in part to the presence of multiple forms of the enzyme with different K_m values and relative activities [11]. At least four apparently distinct forms of phosphodiesterase have recently been demonstrated in extracts of whole larvae of the silkworm *Bombyx mori* [12,13,14]. We have also found multiple forms of phosphodiesterase in cecropia silkmoth fat body, a tissue consisting of only one cell type and having a central role in the insect's carbohydrate, lipid and protein metabolism [15]. Here we report on the separation and characterization of two phosphodiesterases from the fat body of *Hyalophora cecropia* larvae, and on developmental changes in phosphodiesterase activity in this tissue.

Materials and Methods

Materials

Larvae and pupae of the cecropia silkmoth (*H cecropia*) were reared outdoors and in the laboratory as previously described [16].

Aluminum oxide (neutral, activity grade I), cyclic AMP, cyclic GMP, caffeine, theophylline and 5′-nucleotidase (*Crotalus atrox* venom) were from Sigma Chemical Company, St. Louis, Mo. [^{14}C]Adenosine (47 Ci/mol), [^{14}C]-guanosine (49 Ci/mol), cyclic [^{3}H]AMP (24.1 Ci/mmol), cyclic [^{3}H]GMP (4.47 Ci/mmol) were from New England Nuclear, Boston, Mass. The phosphodiesterase inhibitor Ro-7-2956 (4-(3,4-dimethoxybenzyl)-2-imidazolidinone) was a gift from Dr. Paul Greengard.

Before use in assays, the cyclic [^{3}H]nucleotides were purified by applying to alumina columns in 100 mM ammonium acetate, pH 4.0, and eluting first with 3 ml of the same buffer, followed by 1-ml portions of 100 mM ammonium acetate, pH 5.5. The latter fractions, which contained the cyclic nucleotides free of purine bases, nucleosides and 5′-nucleotides, were neutralized with NaOH and dilute Tris · Cl and stored frozen. They gave blank values of less than 2% in phosphodiesterase assays.

Tissue extraction

Fat body was removed from CO_2-anesthetized late fifth instar larvae, rinsed in insect Ringer [17], blotted on filter paper and weighed. Tissue was homogenized with a Potter-Elvehjem homogenizer with a teflon pestle in 2—3 volumes of Tris · Cl buffer (50 mM, pH 8.5) containing 1 mM dithiothreitol. After centrifugation of the homogenate at 1000 × *g* for 15 min, the milky fluid was removed from between a small pellet and a thick, fatty surface layer and centrifuged at 100 000 × *g* for 60 min. The resulting supernatant was concentrated by negative pressure dialysis at 2° C.

Phosphodiesterase assay

Phosphodiesterase was assayed by measuring the [^{3}H]nucleoside derived from cyclic [^{3}H]nucleotide as previously described [10]. Standard reaction mixtures contained 10—50 µl of enzyme (undiluted for homogenates and extracts at millimolar substrate or diluted as much as 1/20th for assays at micromolar substrate), 50 mM Tris · Cl (pH 8.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol and cyclic [^{3}H]nucleotide at various concentrations in a total volume of 200 µl. Reaction tubes were incubated at 30° C usually for 30 min, then immersed in boiling water for 75 s, followed by cooling and treatment with 0.2 units of 5′-nucleotidase for 10 min at 30° C. After addition of 100 µl 0.2 M acetic acid containing the appropriate [^{14}C]nucleoside to monitor recovery, the resulting mixture at pH 4.0 was applied to alumina columns and the nucleoside eluted as previously described [10]. Samples were counted in a toluence/Triton X-100 scintillation fluid. Protein was assayed by the method of Lowry et al. [18] with bovine serum albumin as standard. In kinetic experiments hydrolysis did not exceed 14%, and in other assays 25% of the total substrate.

Results

Separation of phosphodiesterases

Phosphodiesterase activity in crude fat body homogenates was linearly related to the amount of homogenate only in the presence of Triton X-100 (0.2—1.0%), which also caused a slight (up to 30%) increase in total activity. Activity in the soluble supernatant fraction (which generally represented more than 50% of the total activity; see below) was linearly to amount without addition of detergent and was not activated by detergent.

Kinetics suggestive of the presence of multiple forms of phosphodiesterase in larval cecropia fat body have already been reported [10]. Chromatography of a 100 000 × *g* supernatant fraction on Sephadex G-200 achieved separation of two forms of the enzyme (Fig. 1): a first peak eluting in the void volume was accentuated in assays at low (1 µM) substrate concentration, and a second peak eluting at $V_e/V_o = 1.5$—1.7 was predominant in assays at high (1 mM) substrate. The first, presumably low K_m enzyme has similar activity for both cyclic nucleotides and is designated phosphodiesterase I, while the second, presumably high K_m enzyme hydrolyzed cyclic GMP at a rate higher than cyclic AMP and is designated phosphodiesterase II.

Further characterization of and distinction between these kinetic forms were achieved by precipitation with ammonium sulfate (Table I). Nearly all of the

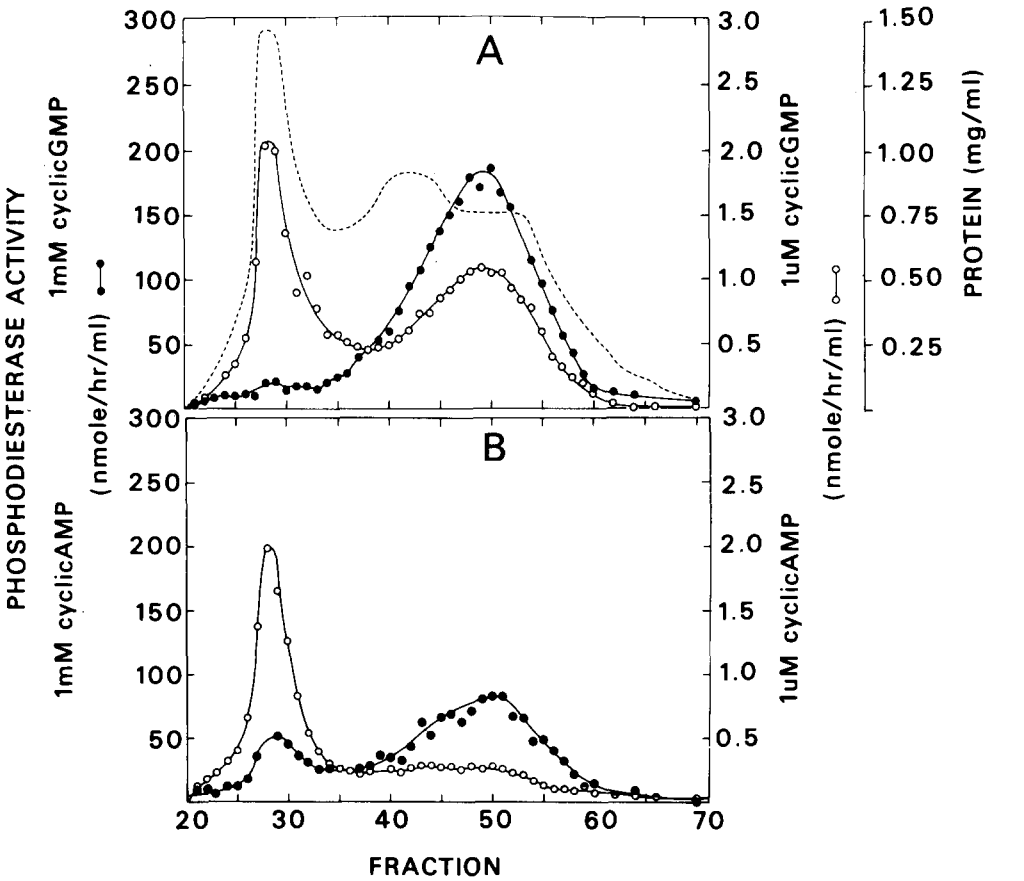

Fig. 1. Elution profiles of cecropia fat body phosphodiesterases on Sephadex G-200. A concentrated extract (1.25 ml, about 30 mg protein/ml) was applied to a 2.5 × 40 cm column of Sephadex G-200 and eluted with 50 mM Tris · Cl, pH 8.5, 0.5 mM dithiothreitol, at 2°C with a flow rate of 25 ml/h. Fractions of 2.0 ml were collected and assayed for phosphodiesterase activity and protein as described in Materials and Methods. The void volume (56 ml) was measured with blue dextran. A, activity on cyclic GMP; B, activity of cyclic AMP. •, activity with 1 mM substrate; ○, activity with 1 μM substrate; - -, protein.

activity from the first Sephadex peak was precipitated by 40% saturation, while that in the second peak precipitated largely in the range 40—70% saturation. The precipitate obtained from the first peak with 30% saturated ammonium sulfate, and that from the second peak with 40—70% saturated were dissolved in Tris · Cl buffer (50 mM, pH 8.5), dialyzed against the same buffer, and stored frozen in droplets at —90°C or in liquid nitrogen, as sources of phosphodiesterases I and II, respectively. Under these conditions there was little loss of activity over a period of several months.

pH optima

Phosphodiesterases I and II, assayed with cyclic GMP as substrate, had very similar pH vs. activity curves, both showing broad optima about pH 8.5, with more than 85% of maximal activity in the range pH 8.0 to 9.0. For standard assay conditions, pH 8.5 was chosen.

TABLE I

AMMONIUM SULFATE PRECIPITATION OF PHOSPHODIESTERASES I AND II AFTER GEL FILTRATION

For phosphodiesterase I, fractions 23—32 were pooled from the Sephadex fractionation of Fig. 1, and for phosphodiesterase II fractions 41—56 were pooled. Ammonium sulfate was added to make 25% saturation and, after 20 min on ice, the suspensions were centrifuged for 10 min at 20 000 × *g*. More ammonium sulfate was added to the decanted supernatants to achieve the desired level of saturation and the process repeated. The sediments were dissolved in the original volume of Tris · Cl (50 mM, pH 8.5) containing 1 mM dithiothreitol, dialyzed, and assayed for activity and protein as described in Materials and Methods.

Ammonium sulfate (% saturation)	Phosphodiesterase I (1 μM cyclic GMP)		Phosphodiesterase II (1 mM cyclic GMP)	
	total activity (nmol/h)	specific activity (nmol/h/mg)	total activity (nmol/h)	specific activity (nmol/h/mg)
	30.3	2.50	3616	181
0—25	16.7	2.23	0	0
26—40	7.9	1.42	127	75
41—55	0.2	0.11	816	175
56—70	0.3	0.27	2395	310
71—85	0	0	248	79

Kinetic properties; inhibitors

Kinetic analysis of the two activities revealed distinct K_m values for each enzyme with each substrate: for phosphodiesterase I, 1.8 μM with cyclic GMP and 10.6 μM with cyclic AMP (Fig. 2A), and for phosphodiesterase II, 125 μM with cyclic GMP and 625 μM with cyclic AMP (Fig. 2B). These K_m values are means from two or more experiments similar to that shown. With phosphodiesterase I, the V for cyclic AMP was approximately 5-fold greater than that for cyclic GMP, whereas phosphodiesterase II gave the same V for both substrates.

A study of the effect of each cyclic nucleotide upon the hydrolysis of the other showed that, with both phosphodiesterases, cyclic AMP competitively inhibited cyclic GMP hydrolysis and cyclic GMP competitively inhibited cyclic AMP hydrolysis. Plotting v^{-1} against [S] at two concentrations of inhibitor [19] gave the following K_i values: for phosphodiesterase I with cyclic GMP as inhibitor 1.6 μM and with cyclic AMP as inhibitor 9 μM; for phosphodiesterase II with cyclic GMP as inhibitor 180 μM and with cyclic AMP as inhibitor 780 μM. For both enzymes, the K_i for each nucleotide as inhibitor approximated to its K_m as substrate, suggesting involvement of the same sites. The methyl xanthines caffeine and theophylline, known inhibitors of phosphodiesterases from a wide variety of sources [11], competitively inhibited cecropia phosphodiesterase I with K_i values of 0.1—0.5 mM, and phosphodiesterase II with K_i values of 1.0—1.5 mM. The compound Ro-7-2956, a more potent inhibitor of mammalian phosphodiesterases than the methyl xanthines [20], at 1 mM inhibited phosphodiesterase I-catalyzed hydrolysis of 2 μM cyclic GMP by 49%, and phosphodiesterase II-catalyzed hydrolysis of 200 μM cyclic GMP by only 25%.

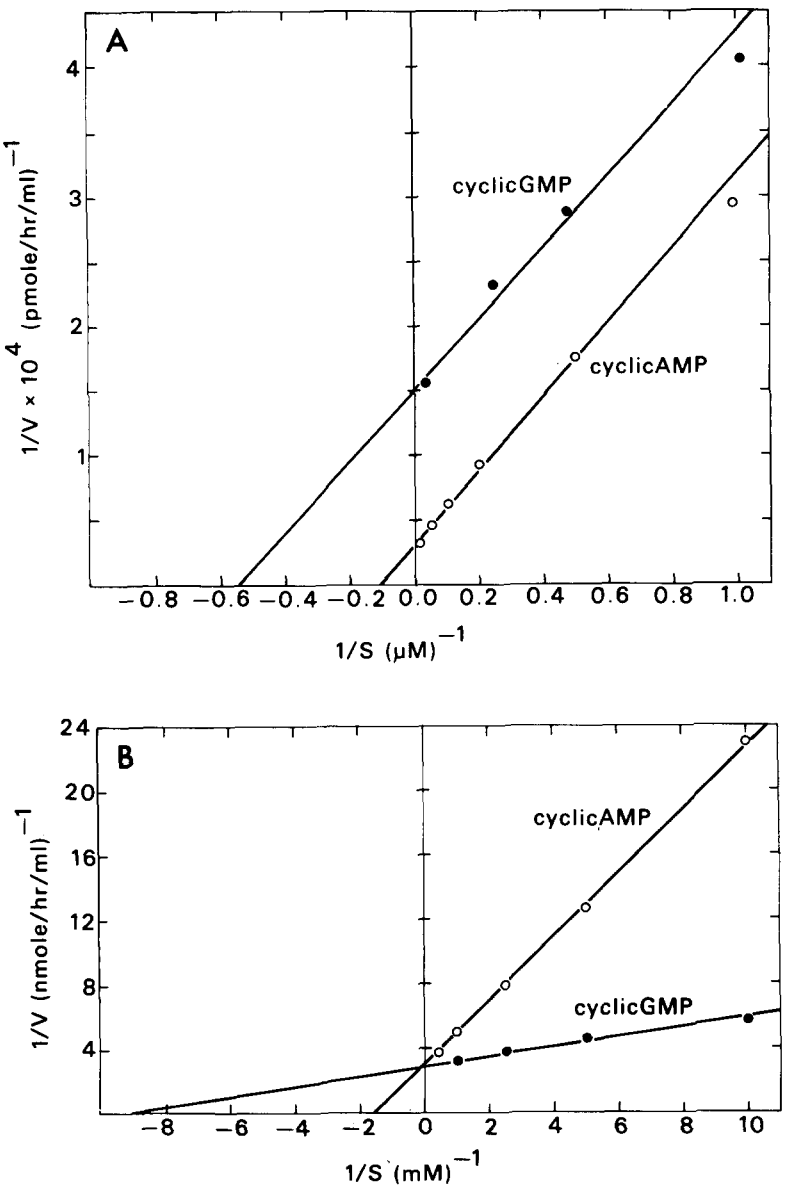

Fig. 2. Line weaver-Burk plots of cyclic nucleotide hydrolysis by fat body phosphodiesterases. A, phosphodiesterase I; B, phosphodiesterase II. ○, cyclic AMP as substrate; ●, cyclic GMP as substrate.

Effects of metal ions and EDTA

Cyclic nucleotide phosphodiesterases usually require a divalent metal ion for maximal activity. The two cecropia fat body enzymes differed in this respect. Phosphodiesterase I activity was stimulated about 2.5 fold by 5—10 mM Mg^{2+}, with half-maximal activation at 100—200 μM (Fig. 3). Similar levels of Co^{2+} and low concentrations of Mn^{2+} activated, though less strongly, while Ca^{2+} and higher concentrations of Mn^{2+} were inhibitory (Table II). The strong inhibition by EDTA suggests that divalent ions are essential, and the activity observed

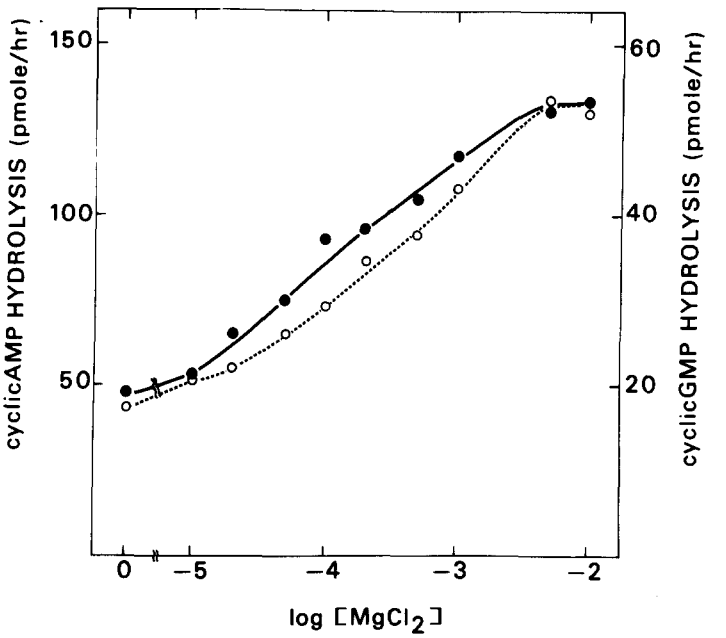

Fig. 3. Effect of $MgCl_2$ on activity of phosphodiesterase I. ○, 5 μM cyclic AMP as substrate; ●, 2 μM cyclic GMP as substrate.

TABLE II

EFFECTS OF DIVALENT METAL IONS AND EDTA ON PHOSPHODIESTERASES OF SILKMOTH FAT BODY

Phosphodiesterase I was the 0—30% saturated ammonium sulfate fraction from the first peak from Sephadex chromatography and was assayed with 2 μM cyclic GMP; phosphodiesterase II was the 40—70% saturated ammonium sulfate fraction from the second Sephadex peak and was assayed with 500 μM cyclic GMP.

Effector	Concentration (mM)	Relative activity	
		phosphodiesterase I	phosphodiesterase II
none	—	100	100
Mg^{2+}	0.05	157	—
Mg^{2+}	0.5	216	102
Mg^{2+}	5.0	272	101
Ca^{2+}	0.05	108	100
Ca^{2+}	0.5	77	99
Ca^{2+}	5.0	54	96
Co^{2+}	0.05	174	99
Co^{2+}	0.5	195	102
Co^{2+}	5.0	205	101
Mn^{2+}	0.05	133	104
Mn^{2+}	0.5	161	89
Mn^{2+}	5.0	41	39
EDTA	1.0	9	97
EDTA	10.0	2	99

with no addition is due to the presence of bound ions in the enzyme preparation. Phosphodiesterase II on the other hand, was neither activated by any added ion nor inhibited by EDTA; only high levels of Mn^{2+} were inhibitory (Table II).

Thermolability; tests for a heat-stable activator

Preincubation of fat body supernatant fraction at 50°C for 5 min reduced phosphodiesterase activity on both 1 μM and 1 mM cyclic GMP by 80—90%, and treatment at 60°C totally abolished activity. This contrasts with the phosphodiesterase of the tobacco hornworm central nervous system, which is stable for at least 10 min at 50°C [21]. Addition of fat body extracts, heated at 80°C for 5 min, to the separated enzymes did not increase activity, and mixing of unheated, dialyzed or undialyzed extracts with the enzymes gave no more than additive activity. EGTA was also without effect on the activity of extracts of separated phosphodiesterases. We were thus unable to find any evidence for a thermostable, Ca^{2+}-dependent activator [22,23].

Subcellular distribution

Phosphodiesterase activity of cecropia larval fat body is distributed in both the particulate and soluble fractions of homogenates. The distribution of activity was assayed at low and high substrate concentrations in fractions derived by differential centrifugation of homogenates after removal of the thick fatty surface layer obtained by centrifugation at 1000 × *g* (Table III). Hydrolysis of cyclic GMP exceeded that of cyclic AMP in all fractions and at both substrate concentrations. At 1 mM cyclic GMP, the activity was distributed in roughly equal propertions in the 1000 × *g* pellet and the 100 000 × *g* supernatant, while comparatively little activity was found in the mitochondrial or microsomal

TABLE III

SUBCELLULAR DISTRIBUTION OF FAT BODY PHOSPHODIESTERASE ACTIVITY AT HIGH AND LOW SUBSTRATE CONCENTRATIONS

Larval fat body homogenates prepared in Tris · Cl (50 mM, pH 8.5) containing 1 mM dithiothreitol with or without 0.25 M sucrose, were centrifuged at 1000 × *g* for 15 min, the fat removed, the supernatant decanted, and the sediment washed three times. The 1000 × *g* supernatant was centrifuged at 20 000 × *g* for 20 min and the sediment washed three times. The resulting supernatant was centrifuged at 100 000 × *g* for 90 min and the supernatant decanted. All sediments were suspended in the original volume of buffer. The absence of sucrose in the homogenizing buffer made no difference in the distribution of activities. Values are averages from three separate experiments. Activities for the homogenate were obtained after removal of the fat layer.

Fraction	Phosphodiesterase activity (pmol/min/mg wet wt.)			
	cyclic AMP		cyclic GMP	
	1 mM	0.15 μM	1 mM	0.16 μM
Homogenate	24.4	0.092	62.4	0.123
1 000 × *g* sediment	7.3	0.007	29.9	0.022
20 000 × *g* sediment	1.7	0.012	6.8	0.026
100 000 × *g* sediment	2.6	0.002	3.8	0.010
100 000 × *g* supernatant	15.0	0.057	24.4	0.073

TABLE IV

DEVELOPMENTAL CHANGES IN CYCLIC NUCLEOTIDE PHOSPHODIESTERASE OF SILKMOTH FAT BODY

Substrate	Phosphodiesterase activity (pmol/min/mg wet wt.) [a]					
	Stage of development [b]					
	L	S-1	S-6	E	P	PA
Cyclic GMP						
1 μM	1.09 ± 0.10	0.76 ± 0.04	0.98 ± 0.14	0.57 ± 0.05 [c]	0.037 ± 0.0008 [d]	0.170 ± 0.017 [d]
1 mM	89 ± 15	96 ± 11	105 ± 21	126 ± 15	3.7 ± 0.6 [d]	17.3 ± 2.4 [d]
Cyclic AMP						
1 μM	0.50 ± 0.06	—	—	—	0.054 — 0.002 [e]	0.194 ± 0.022 [d]

[a] Means ± S.E.M. (N = 4.6).

[b] Stages: L, mature fifth-instar larvae; S-1, larvae on the first day of spinning; S-6, pharate pupae 6 days after spinning; E, fresh pupae after ecolysis but before tanning; P, pupae 30 or more days old; PA, early pharate (= developing) adults.

[c] $P < 0.014$ when compared to the preceding stage of development.

[d] $P < 0.01$ when compared to the preceding stage of development.

[e] $P < 0.01$ when compared to the larval stage.

pellets. At 0.16 μM cyclic GMP, however, there was proportionately less activity in the 1000 × *g* pellet and more in the mitochondrial pellet, while most of the activity was in the soluble supernatant. Activity with cyclic AMP showed quite similar distribution.

The fatty surface layer also contained significant activity (10—20% of that of the total homogenate), measured at 1 μM or 1 mM substrate. Some of this activity was particulate and, after resuspension, could be sedimented at low speed. Addition of Triton X-100 (0.2—1.0% final concentration) increased the total fat layer activity by 10—30%, and also produced comparable activation in whole homogenates, although it had no effect on the activity of separated phosphodiesterases I and II.

Developmental changes in activity

Homogenates of fat body from animals at a series of stages of development were assayed at 1 μM and 1 mM cyclic GMP (and in some cases also 1 μM cyclic AMP) in the presence of 0.3% Triton X-100 (Table IV). Activities were highest in the larval stages, fell to low levels in the pupa, and increased again during pharate adult development. Mixed larval and pupal homogenates showed either additive activity (at 1 mM substrate) or only a 22% decrease in larval activity (1 μM substrate), thus indicating that the decrease at pupation was not due to the presence of an inhibitor.

Discussion

In nearly every animal tissue that has been examined by appropriate methods, cyclic nucleotide phosphodiesterase has been found in at least two forms, characterized by K_m values that differ by at least an order of magnitude [11,24,25,26]. Among the insects, phosphodiesterase activity has been demonstrated in many organs and tissues of various species [20,27—31].

From extracts of whole *Bombyx* silkworm larvae, no less than four phosphodiesterases with distinctive properties have been separated, three favoring cyclic AMP as substrate and one favoring cyclic GMP [12,13,14], but their tissue origins are not known. Phosphodiesterases have recently been partially characterized from hornworm central nervous system tissue [20] and cricket male accessory gland [31]. Phosphodiesterase from fat body, a tissue with metabolic and biosynthetic functions of especial interest analogous to those of the vertebrate liver and adipose tissue [15], has, however, not previously been characterized.

Cecropia silkmoth fat body contains two distinct, soluble phosphodiesterases which differ considerably in their apparent molecular size, solubility in ammonium sulfate solutions and divalent metal ion dependence, as well as kinetic properties. Phosphodiesterase I, with K_m values in the micromolar range, has both K_m and V about 5-fold greater for cyclic AMP than for cyclic GMP; thus, at physiological substrate levels, which are likely to be below the K_m values, it would hydrolyze both substrates with about equal velocity. Phosphodiesterase II, with K_m values higher but again about 5-fold greater for cyclic AMP than for cyclic GMP, and with equal V for both substrates, would be about 5-fold more active against cyclic GMP than cyclic AMP at low levels. As a consequence of

these properties and the relatively greater activity of phosphodiesterase II than phosphodiesterase I in larval fat body extracts, these extracts have greater capacity to hydrolyze submicromolar levels of cyclic GMP than cyclic AMP. The activity under these conditions is about equally divided between the two enzymes (see Fig. 1A and B, areas under the curves).

Whereas phosphodiesterase I, like the vertebrate phosphodiesterases that have been described, requires a divalent metal ion for activation, cecropia fat body phosphodiesterase II is neither activated by such ions nor inhibited by EDTA. Insensitivity to divalent metal ions and EDTA has also been reported for some forms phosphodiesterase from silkworm larvae [12] and hornworm nervous tissue [20], and thus appears to be a characteristic of certain insect phosphodiesterases not yet reported, so far as we are aware, for any phosphodiesterase of vertebrate origin. Another apparently regulatory property common to many vertebrate phosphodiesterases, activation by a heat-stable Ca^{2+}-dependent protein [22,23,32] could not be demonstrated for either of our enzymes, even though the activator protein has been found in insect tissue [33]. Yet another point of difference from certain vertebrate phosphodiesterases is the relatively weak inhibition by the compound Ro-7-2956, which is a 40-fold more powerful inhibitor of rat erythrocyte phosphodiesterase than theophylline [19]. Insensitivity to similar inhibitors has been described for the hornworm nervous tissue phosphodiesterase [20].

The subcellular distribution of phosphodiesterase activity varies among the different insect tissues and species that have been examined [20,28,30,31], but preponderance in the low speed pellet and the soluble fraction, with little activity in the mitochondrial and microsomal fractions, has been reported in some cases [30,31]. This is the pattern that we find for phosphodiesterase II. While our attention has been centered on the soluble enzymes, recent evidence for hormonal regulation of particulate phosphodiesterases of mammalian adipose tissue and liver [34,35,36] suggests that the particulate activity of fat body may deserve further investigation.

During development of the cecropia silkmoth, fat body phosphodiesterase activity, high in the larva, falls to very low levels in the pupal stage and then rises again during development of the adult. Some decline in phosphodiesterase activity in the pupal stage has been reported for several other insects [27,37,38, 39]. The extreme drop in activity in the cecropia pupa is associated with the state of diapause or arrested development, in which many metabolic and biosynthetic activities are suppressed [40]. Yet, it is interesting to note that adenyl and guanyl cyclases do not undergo marked decrease in the diapausing cecropia pupa [9].

The roles of cyclic AMP and cyclic GMP in hormonal and other regulation in insect fat body are not yet clear. However, the presence of phosphodiesterases acting on both cyclic nucleotides, along with active adenyl and guanyl cyclases [9] having some properties in common with those of vertebrate tissues, suggests essential similarity of the insect cyclic nucleotide systems to those of vertebrates. Although cyclic GMP and associated enzymes are sometimes quantitatively more abundant in insect tissues than in those of vertebrates, no further support has been obtained for the suggestion that in certain insect tissues cyclic GMP may carry out some of the roles performed elsewhere by cyclic AMP [41].

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