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PROPERTIES OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA*

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Summary

The properties of cyclic nucleotide phosphodiesterase were studied in soluble and particulate fractions from the central nervous system of *Manduca sexta* (Lepidoptera: Sphingidae). It was determined that: (1) The highest levels of phosphodiesterase occur in nervous tissue. (2) The total and specific enzyme activities of larval and adult brains are greater than those of the remaining ganglia. (3) Specific central nervous system phosphodiesterase activities of the adult are lower than those of the larva, but both protein and total phosphodiesterase contents are considerably greater in the adult central nervous system. (4) Mg^{2+} is not absolutely required for either cyclic AMP-phosphodiesterase or cyclic GMP-phosphodiesterase activity. (5) Phosphodiesterase is inhibited by a variety of physiological and non-physiological compounds, nucleoside triphosphates being particularly effective. Some potent inhibitors of mammalian phosphodiesterase are comparatively ineffective toward *Manduca sexta* phosphodiesterase. (6) Kinetic analyses of soluble and particulate phosphodiesterase revealed non-linear double-reciprocal plots for the hydrolysis of both cyclic AMP and cyclic GMP, with Michaelis constants of approximately 10 μM and 20 μM. (7) The hydrolysis of both cyclic nucleotides appears in part to be the function of a single enzyme or related enzymes in the insect central nervous system. It follows that the intracellular level of one cyclic nucleotide may influence the concentration of the other by inhibiting its degradation.

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Abbreviation: Ado-*P*-CH_2-*P*-*P*,α,β-methyleneadenosine-5′-triphosphate.

Introduction

In most cells the concentration of cyclic AMP is determined by the relative activities of hormone-sensitive adenylate cyclase (EC 4.6.1.1) and of one or more nucleoside 3′,5′-monophospate phosphodiesterases (EC 3.1.4.17). Similarly, the intracellular level of cyclic GMP is governed by the relative activities of phosphodiesterase and guanylate cyclase (EC 4.6.1.2). Phosphodiesterases are present in both cytosolic and particulate fractions of the cell [1—3]. In rat liver [4], one enzyme is soluble and specific for cyclic GMP, another particulate and specific for cyclic AMP, and the third soluble and capable of degrading both cyclic AMP and cyclic GMP. In many tissues it appears there exist at least 2, and often more, forms of phosphodiesterase [1,3,5,6].

Phosphodiesterase may be viewed as a key regulatory molecule responsible in part for the magnitude and duration of hormone action. Regulation of phosphodiesterase activity may occur by inhibition by nucleoside triphosphates and pyrophosphate [7], allosteric modulation [5,8], protein—protein interactions [9,10], protein—lipid interactions [11], and induction [12,13]. Mammalian brain preparations have proved convenient systems for the study of phosphodiesterase because this enzyme has been found to be much more abundant in brain than in any other tissue [14—16]. We report here some of the results of an investigation of the properties of phosphodiesterase in central nervous tissues of the insect, *Manduca sexta*.

Materials and Methods

Materials

[^{3}H] Adenosine 3′,5′-cyclic monophosphate 24 Ci/mmole) and [^{3}H] guanosine 3′,5′-cyclic monophosphate (4 Ci/mmole) were obtained from New England Nuclear and purified (> 99.9%) on microcrystalline cellulose chromatographic plates (Applied Science Laboratories) using 2-propanol/NH_4OH/H_2O (14 : 3 : 3, v/v/v) as a solvent. Unlabeled cyclic AMP and cyclic GMP in neutral 10 mM Tris—HCl buffer were mixed with lyophilized, tritiated nucleotide to yield a $5.0 \cdot 10^{-4}$ M stock solutions of the following specific activities: cyclic AMP, 400 to 600 dpm/pmole; cyclic GMP, 100 to 200 dpm/pmole.

Anion exchange resin (Bio-Rad Laboratories AGl-X2, 200—400 mesh) was washed twice in 0.5 M NaOH, acetone, and 0.05 M HCl, finally rinsed with glass-distilled water to pH 6, and stored in glass-distilled water. Lyophilized snake venom (*Ophiophagus hannah*) was obtained from Sigma. 4-(3,5-Dimethoxybenzyl)-2-imidazolidinone (Ro20-2926) was kindly provided by Dr Herbert Sheppard of Hoffmann-La Roche, Department of Pharmacology and 1-ethyl-4-(isopropylidenehydrazine)-1H-pyrazole-(3, 4-b)-pyridine-5-carboxylic acid, ethyl ester (SQ20009) was supplied by Dr Sidney Hess of the Squibb Institute for Medical Research.

M. sexta cultures

M. sexta eggs were graciously supplied by Mr A.H. Baumhover, U.S.D.A., Oxford Research Station, Oxford, North Carolina. Larvae were reared according to the procedures of Yamamoto [17]. Fifth-instar larvae of both sexes were

selected for dissection. Some larvae were allowed to develop to the adult stage in wooden pupal cells [18] and collected between 12 and 36 h after emergence.

Central nervous system dissections and enzyme preparations

The *M. sexta* larval nerve cord is similar to *Galleria mellonella* [19] and consists of a two-lobed brain, a suboesophageal, three thoracic, and eight abdominal ganglia, with the last two abdominal ganglia being fused. During larval-to-adult neurometamorphosis there occurs a pronounced shortening of the interganglionic connectives and a fusion of several ganglia yielding seven ganglionic masses in the adult central nervous system.

Larval and adult insects were chilled on ice, pinned ventral surface down in a dissection dish, and a longitudinal dorsal incision made. During the dissection under a microscope the body cavity was filled with an ice-cold saline solution [20]. All lateral connectives of the ventral nerve cord were transected with iridectomy scissors, the excised nerve cord was freed from adhering non-neural tissues, thoroughly rinsed with saline followed by glass-distilled water, and immediately frozen on dry ice. Homogenizations were performed by motor-driven pestle in all-glass micro tissue grinders (Kontes Duoll 20) as indicated in the result section.

Ammonium sulfate precipitation of soluble phosphodiesterase

Phosphodiesterase was precipitated from the 10 000 × *g* for 20 min larval supernatant by adding an equal volume of a saturated ammonium sulfate solution dropwise over a 3 min period. This mixture was stirred on ice for 30 min, allowed to stand an additional 15 min in the cold, then centrifuged at 20 000 × *g* for 20 min. The pellet was suspended in 10 mM Tris buffer (pH 7.5) containing 1 mM $MgSO_4$ and dialyzed for 16 h at 0—4°C against the same buffer. In experiments designed to investigate bivalent metal ion requirements of phosphodiesterase, magnesium was omitted from all buffers. After dialysis, the slightly turbid solution was centrifuged at 10 000 × *g* for 10 min, and the precipitate was discarded. The supernatant, termed the (0—50)AS fraction, was divided into small aliquots, frozen in liquid nitrogen, and stored at −20°C until use. The recoveries of soluble enzyme activity in the (0—50)AS fraction were 100% for cyclic AMP-phosphodiesterase and 76% for cyclic GMP-phosphodiesterase. Although the purification factors were small (60% for cyclic AMP-phosphodiesterase and 22% for cyclic GMP-phosphodiesterase), ammonium sulfate precipitation removes the enzyme activities from the original environment of cellular salts, miscellaneous nucleotides, and other potentially inhibitory substances of the cytosol.

Cyclic nucleotide phosphodiesterase assays

Two radiometric assays were used to quantitate cyclic AMP-phosphodiesterase. Assay I was employed for relatively rapid analyses not requiring extreme accuracy or precision. This assay consists of a modification of the two-step methods of Butcher and Sutherland [14] and Thompson and Appleman [2]. The final reaction mixture contained 50 mM Tris—HCl (pH 7.5), 5 mM $MgSO_4$, 50 μM cyclic [^{3}H]AMP unless noted otherwise, and less than 50 μg of

enzyme protein in a total volume of 0.20 ml. After equilibration of the enzyme for 10 min at 30°C, the reaction was initiated by the addition of cyclic [^{3}H]AMP, the mixture was incubated at 30°C in a shaking water bath for 10 min and the reaction terminated by boiling for 3 min. Snake venom (*Ophiophagus hannah*, 0.1 mg in 0.1 ml) was added to the cooled reaction mixture, well mixed, and incubated for another 30 min at 30°C to effect quantitative conversion of 5′-AMP to adenosine. This latter reaction was terminated by the addition of 1.0 ml of a 1 : 2 (w/v) slurry in water of AGl-X2 anion exchange resin, which preferentially binds cyclic [^{3}H]AMP and leaves [^{3}H]adenosine in the aqueous phase. The slurry was agitated at room temperature for a minimum of 10 min, and after centrifugation at 1000 × *g* for 5 min, an aliquot (0.1 ml) of the supernatant was taken for liquid scintillation counting in 10 ml of Aquasol (New England Nuclear).

The composition of the reaction mixture for Assay II is identical to that described for the initial step of Assay I. After 10 min of incubation at 30°C, the reaction mixture was boiled for 3 min, cooled, and 20 μl of a carrier solution containing 0.01—0.05 μmole each of cyclic AMP, 5′-AMP, and adenosine was added. A 25 μl aliquot of the resultant solution was spotted on chromatography grade Whatman No. 1 paper. Chromatograms were developed for 16—24 h in an ascending fashion at room temperature using 1.0 M ammonium acetate—95% ethanol (3 : 7, v/v). Observed R_f values were 0.19 to 5′-AMP, 0.48 for cyclic AMP, and 0.65 for adenosine. The spots were cut and extracted with 3.0 ml of 0.01 M HCl. After incubation with intermittant shaking for 15 min at 80°C, an aliquot of the HCl extract was counted in 15 ml of Aquasol. Better than 99% of the radioactivity in the spots was extracted by this simple one-step procedure. Since both 5′-[^{3}H]AMP and [^{3}H]adenosine were formed, it was necessary to determine the radioactivity in both products to accurately measure enzyme activity. 5′-Nucleotidase is largely particulate in the *M. sexta* central nervous system in some instances up to 50% of the 5′-AMP product of phosphodiesterase was hydrolyzed to adenosine in the course of the standard assay. Blank corrections for both 5′-AMP and adenosine were established at every concentration of cyclic AMP tested. Units of cyclic AMP-phosphodiesterase activity were defined as the sum of the pmoles of 5′-AMP and adenosine formed per min at 20°C under the assay conditions specified.

Cyclic GMP-phosphodiesterase was assayed via the above two methods. The initial concentration of the cyclic [^{3}H]GMP substrate was 50 μM for routine assays. For paper chromatographic separations, 0.05 μmole each of cyclic GMP, 5′-GMP, and guanosine were added to the boiled incubation mixture before application to Whatman No. 1 paper. Observed R_f values in the solvent system described were 0.12 for 5′-GMP, 0.40 for cyclic GMP, and 0.58 for guanosine. The presence of considerable 5′-GMP-phosphohydrolase activity in nerve cord samples made it essential to quantitate both 5′-[^{3}H]GMP and [^{3}H]guanosine. Units of cyclic GMP-phosphodiesterase activity were defined as the sum of the pmoles of 5′-GMP and guanosine formed per min at 30°C.

In most assays the relative levels of enzyme protein and cyclic nucleotide substrate were chosen so that less than 15% of the substrate was consumed at the end of the incubation period. Since phosphodiesterase activity was very sensitive to variations in pH, the volume of enzyme protein assayed was at most

one-tenth to one-fifth of the total incubation volume, thus minimizing the perturbation of the reaction mixture's buffering capacity. Blanks for each experiment were performed in triplicate and were of the following nature: (1) omission of enzyme, (2) heat-inactivated enzyme, or (3) addition of ^{3}H-labeled substrate to a boiled reaction mixture. All experimental data were corrected for an averaged blank value.

Assay II was used in most instances because of the greatly increased accuracy, sensitivity, reproducibility, and also because estimates of both phosphodiesterase and nucleotidase were obtained. Replicate samples assayed by the paper chromatographic method agreed within 0.5% or better, whereas those measured by Assay I often varied 5—10%. 5′-Nucleotide backgrounds observed using Assay II were 0.36% and 0.40% for cyclic AMP and cyclic GMP, respectively. Estimates of phosphodiesterase activity obtained using Assay I were 3—7-fold lower than those found with Assay II chromatographic technique. At an initial cyclic nucleotide concentration of 50 μM in the presence of up to 50 μg of enzyme protein, the hydrolysis of both cyclic AMP and cyclic GMP was linear with protein concentration and time through at least 10 min of incubation, as measured by either Assay I or II.

Results

Miscellaneous properties of M. sexta central nervous system phosphodiesterase

For all nerve cord samples examined by Assay II over a wide range of substrate concentrations, the sum of the radioactivities found in the 5′-nucleotide, nucleoside and unhydrolyzed cyclic nucleotide substrate sections of the chromatogram were equal to the radioactivity of the cyclic nucleotide substrate added. The nucleosides were produced only from the 5′-nucleotides in a time- and concentration-dependent manner. These findings suggest that the sole mechanism of degradation of both cyclic AMP and cyclic GMP in the *M. sexta* nervous system is through hydrolysis to 5′-AMP and 5′-GMP, respectively.

The cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase activities of the *M. sexta* larval central nervous system were very nearly equally distributed among soluble and particulate fractions following homogenization of nerve cords in hypotonic buffer. Storage of homogenates or 10 000 × *g* supernatants for as long as 3 months at −20°C led to no detectable loss of enzymatic activity. In fact, repetitively freezing and thawing homogenates prior to assay resulted in a 10—20% increase in both cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase activities. Brief sonication of homogenates did not solubilize particulate-associated activity nor did it affect the soluble activity. Phosphodiesterase activity was stable to heating at 50°C for 10 min but was destroyed after boiling for 3 min. Incubation of larval nerve cord homogenates on ice for 10 min with Triton X-100 (0.50%, v/v) prior to assay resulted in about a 30% increase in both phosphodiesterase activities. Since Triton had no effect on soluble phosphodiesterase, it may be assumed that this detergent unmasks activity in particulate samples by solubilizing membrane or organelle-associated phosphodiesterase.

NaCl and KCl at 50 mM were without appreciable effect on cyclic AMP-phosphodiesterase or cyclic GMP-phosphodiesterase. Bovine serum albumin

(0.1 mg/ml final concentration) or glycerol at 1% (v/v) in the incubation mixture had no effect on activity. A 15% loss in activity of cyclic AMP-phosphodiesterase activity occurs during 40 min storage on ice but no loss in cyclic GMP-phosphodiesterase activity resulted. This selective loss was not due to proteolytic enzymes since incubation with trypsin (1 μg per 100 μg homogenate protein, 5 min at 30° C) had no effect.

Relative phosphodiesterase activities in various M. sexta tissues

Since phosphodiesterase is more abundant in mammalian brain [14—16] than non-neural and peripheral nervous tissue, it was of interest to compare its levels in various *M. sexta* tissues. As is indicated in Table I, cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase activities were found in each of the insect tissues examined. The ratio of cyclic GMP to cyclic AMP activity varied greatly among tissues, as did the relative levels of activity in soluble and particulate fractions. By far the highest specific phosphodiesterase activities were found in the larval and adult nerve cords. Nerve cords contained high levels of both phosphodiesterases while muscle preparations were relatively enriched in cyclic AMP-phosphodiesterase. The specific activity of cyclic AMP-phosphodiesterase in *M. sexta* central nervous system homogenates is of the same order of magnitude as that in mammalian brain preparations. Comparison of the activities between homogenate and supernatant from a given tissue in Table I is indicative of the fraction of enzyme activity that is soluble since equal volumes were assayed.

TABLE I

PHOSPHODIESTERASE ACTIVITIES IN VARIOUS *M. SEXTA* TISSUES

All tissues were throughly homogenized in glass tissue grinders in 50 mM Tris—HCl (pH 7.5) containing 1 mM $MgSO_4$. A portion of the homogenate was immediately centrifuged at 34000 × *g* for 30 min. Both the homogenates and supernatants were assayed for activity in the presence of 50 μM cyclic [^{3}H] AMP or cyclic [^{3}H] GMP according to Assay II. Homogenate specific activities were determined only for the central nervous system preparations.

Tissue	Phosphodiesterase activity (units/reaction mixture)		Specific activity of phosphodiesterase (units/mg protein)	
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
Larval central nervous system, homogenate	248	316	4960	6360
Larval central nervous system, supernatant	163	254	3480	5430
Larval fat body, homogenate	73	142	—	—
Larval fat body, supernatant	14	12	1200	1060
Larval haemolymph, homogenate	70	164	—	—
Larval haemolymph, supernatant	79	177	690	1540
Larval gut, homogenate	78	50	—	—
Larval gut, supernatant	67	62	1200	1110
Larval muscle, supernatant	50	10	2100	420
Adult central nervous system, homogenate	268	269	2860	2860
Adult central nervous system, supernatant	173	136	5190	4060
Adult muscle, homogenate	182	37	—	—
Adult muscle, supernatant	80	8	1900	200

Phosphodiesterase activities in the larval and adult central nervous system

In Table II are compiled representative data from a number of experiments with the entire central nervous system from both larval and adult *M. sexta*. Activities measured in fresh, unfrozen larval nerve cords (experiment 4) compared favorably with those determined in tissues frozen on dry ice before homogenization. The greater activities measured in samples homogenized in hypotonic buffer probably resulted from osmotic rupture of subcellular organelles. With the exception of adult nerve cord supernatants, cyclic GMP was more rapidly hydrolyzed than cyclic AMP. A considerable fraction of the phosphodiesterase in both tissues is sedimentable, i.e. associated with particulate fractions. The specific enzyme activities of adult homogenates were somewhat lower than those from larva, but both total cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase were greater in the adult.

TABLE II

PHOSPHODIESTERASE ACTIVITIES IN THE *M. SEXTA* LARVAL AND ADULT CENTRAL NERVOUS SYSTEM

Nerve cords were homogenized in either neutral 50 mM Tris—HCl—1 mM $MgSO_4$ (hypotonic buffer) or 50 mM Tris—HCl—1 mM $MgSO_4$—0.32 M sucrose (hypertonic buffer). Homogenates were centrifuged at 700 × *g* for 10 min, 10000 × *g* for 20 min, 34000 × *g* for 30 min, or 105000 × *g* for 2 h, as indicated. Phosphodiesterase was measured according to Assay II. N.D., not determined.

Experiment	Tissue	No. of central nervous system	Homogenizing buffer*	Enzyme source	Phosphodiesterase			
					Specific activity** (units/mg protein)		Total activity (units/central nervous system)	
					Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
1a	Larval central nervous system	55	H+	Homogenate	2837	4900	867	1497
2a		60	H+	Homogenate	2744	5216	748	1421
3a		15	H−	Homogenate***	6014	6183	2053	2111
3b		15	H−	Homogenate†	4674	5973	1595	2039
4		4	H−	Homogenate††	5589	5783	1906	1972
1b		55	H+	700 × *g* supernatant	4227	5870	805	1118
2b		60	H+	10000 × *g* supernatant	3415	5066	533	791
5		20	H−	34000 × *g* supernatant	5319	N.D.	569	N.D.
1c		55	H+	105000 × *g* supernatant	3517	6717	368	704
6a	Adult central nervous system	11	H−	Homogenate†††	2856	2860	2439	2442
6b		11	H−	10000 × *g* supernatant	5192	4060	1495	1169

* H+, hypertonic; H−, hypotonic homogenizing buffer.
** Both substrates present initially at 50 μM.
*** Assayed immediately after homogenization.
† Same homogenate assayed 40 min later.
†† Fresh, unfrozen nerve cords; protein content not determined; specific activity calculated using mean protein/central nervous system value found in other experiments.
††† Frozen-thawed 1 × before assay.

Phosphodiesterase activities in larval and adult brains

Examination of phosphodiesterase activity in larval brain and each ganglion indicated more activity in brain (150 units per brain) than any of the other ganglia (70—90 units per ganglion). In ganglia other than brain cyclic GMP-phosphodiesterase activity was greater than cyclic AMP-phosphodiesterase activity.

If the brain homogenate is prepared in hypertonic buffer the addition of 0.5% Triton results in an increase in cyclic AMP-phosphodiesterase activity but has no effect on cyclic GMP-phosphodiesterase activity (Table III). This is in contrast to results with the whole central nervous system where the activity of both enzymes is increased 30%. If the brain homogenates are prepared in hypotonic media the cyclic GMP-phosphodiesterase activity is less (6300 units/mg protein) than when prepared in hypertonic buffer (Table III). This suggests the possibility of the existence of a phosphodiesterase specific for cyclic AMP. In addition, the washed 34 000 × *g* pellet contained much higher specific phosphodiesterase activity than the supernatant fraction. It is of interest that the rates of hydrolysis of both cyclic nucleotides are equal in the 34 000 × *g* pellet from brain; in contrast cyclic GMP activity is twice that of cyclic AMP activity in the pellets from the bulk central nervous system (Table IV). The fraction of particulate-associated phosphodiesterase is greater in the brain than in the entire central nervous system.

During larval to adult development, total cyclic AMP-phosphodiesterase activity per brain increases 7-fold, from 150 units to 1050 units. A 4-fold increase in total cyclic GMP-phosphodiesterase activity in brain occurs, from 160 units to 700 units. This increase in activity is likely due to an increase in the absolute number of synapses and/or with the magnitude of synaptic activity.

TABLE III

SOLUBLE AND PARTICULATE PHOSPHODIESTERASE ACTIVITIES IN LARVAL BRAIN HOMOGENATES PREPARED IN HYPERTONIC BUFFER

18 larval brains were dissected, collected on dry ice, and homogenized in 150 µl of 10 mM Tris—HCl (pH 7.5)—0.32 M sucrose—1 mM $MgSO_4$. An aliquot of the homogenate was incubated at 0°C with 0.5% Triton X-100 for 10 min, and the remaining homogenate was centrifuged at 34000 × *g* for 30 min. The pellet was washed once by suspension in the above buffer, collected by centrifugation again, then resuspended in 100 µl of the same. The phosphodiesterase activity of all fractions was measured according to Assay II.

Enzyme source	Total protein of 18 brains (µg)	Specific activity of phosphodiesterase		Ratio of specific activities (cyclic GMP/ cyclic AMP)
		Cyclic AMP	Cyclic GMP	
Homogenate	383	5321	7633	1.43
0.5% Triton* + homogenate	—	7266	7490	1.03
34000 × *g* supernatant	162**	1172	3168	2.70
Washed 34000 × *g* pellet	120**	8154	8212	1.0

* Final concentration of Triton in the reaction mixture was 0.05%

** The total protein recovered in the 34000 × *g* supernatant and washed pellet was less than the protein content of the homogenate because protein was discarded with the buffer used to wash the 34000 × *g* pellet.

TABLE IV

PHOSPHODIESTERASE ACTIVITIES IN CRUDE SUBCELLULAR FRACTIONS FROM THE *M. SEXTA* LARVAL CENTRAL NERVOUS SYSTEM

55 larval nerve cords were dissected over a 3 day period and frozen at −80°C, then homogenized in 1.2 ml of 10 mM Tris—HCl (pH 7.5)—1 mM $MgSO_4$—0.32 M sucrose—1 mM dithiothreitol and centrifuged at 700 × *g* for 10 min, 10000 × *g* for 20 min and 105000 × *g* for 60 min at 0—4°C in polycarbonate screw-capped tubes under N_2. Each particulate fraction, with the exception of the 105000 × *g* pellet, was washed once and re-centrifuged before suspension and assay.

Fraction	Fraction volume (ml)	Protein (g/10 μl)	Volume assayed (μl)	pmoles in 10 min per reaction mixture				Specific activity of phosphodiesterase	
				Adenosine	5′-AMP	Guanosine	5′-GMP	Cyclic AMP	Cyclic GMP
Homogenate 700 × *g*	2.4	70	10	416	1570	714	2716	2837	4900
supernatant 700 × *g*	2.38	44	10	267	1594	227	2355	4227	5870
pellet 10000 × *g*	2.15	28.6	20	182	483	152	886	1163	1813
pellet 105000 × *g*	1.0	12	20	474	1233	488	2987	7112	14483
pellet 105000 × *g*	0.40	29	20	1443	1671	1371	4471	5369	10072
supernatant	2.88	20	20	75	1332	60	2627	3517	6718

Distribution of phosphodiesterase among soluble and particulate fractions

The highest specific cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase activities were found in the 10 000 × *g* and 105 000 × *g* pellets (Table IV). From the standpoint of comparative neurochemistry this finding appears significant since similarly prepared fractions from mammalian brain are rich in phosphodiesterase activity and contain synaptic membranes, vesicles, and small nerve endings [21]. It was calculated that 50% of the total homogenate cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase activity is sedimentable and that 36% is found in the 10 000 × *g* and 105 000 × *g* pellets.

In addition, it was found that 5′-nucleotidase was absent in the 105 000 × *g* supernatant, particularly high in the 105 000 × *g* pellet, and present in all other fractions.

At a cyclic nucleotide substrate concentration of 50 μM, cyclic GMP was more rapidly hydrolyzed than cyclic AMP by all the nerve cord fractions. As noted in rat brain and liver preparations if the concentration of cyclic nucleotide substrate is reduced the ratio of cyclic GMP/cyclic AMP activity approaches one.

Kinetic properties of phosphodiesterase

Several preparations from the larval central nervous system were assayed for cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase at concentrations in the range 1 to 500 μM. For each enzyme sample it was established that the hydrolysis of substrate was linear at least through 10 min of

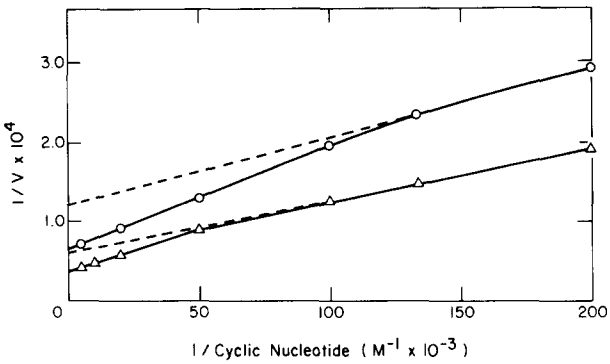

Fig. 1. Lineweaver—Burk plot for the hydrolysis of cyclic AMP and cyclic GMP by a 10 000 × *g* (mitochondrial) pellet from the larval central nervous system. Assay II was employed. Units of reaction velocity, *V*, are pmoles of substrate consumed per min per mg protein. ○———○, cyclic AMP; △———△, cyclic GMP.

incubation, and analysis of the fixed time (10 min) assay data specified *V*, the initial specific reaction velocity (pmole/min/mg protein). Lineweaver—Burk double-reciprocal plots for the hydrolysis of both cyclic AMP and cyclic GMP were in every case distinctly nonlinear (Fig. 1). A discontinuity in slope was generally observed at a substrate concentration of about 10 to 20 μM. Two apparent Michaelis constant (K_m) values and two *V* values were derived by extrapolation of the locally linear segments of Lineweaver—Burk plots. Anomalous double-reciprocal plots such as these have been reported by others in a variety of crude and partially purified cyclic AMP-phosphodiesterase preparations [22—26].

TABLE V

KINETIC CONSTANTS FOR THE HYDROLYSIS OF CYCLIC AMP AND CYCLIC GMP CATALYZED BY VARIOUS LARVAL CENTRAL NERVOUS SYSTEM PREPARATIONS

Phosphodiesterase preparations were assayed in the presence of 1—500 μM substrate according to Assay II. Backgrounds were determined for every sample at each substrate concentration tested, and substrate consumption was generally less than 15%. Kinetic constants were graphically determined from slopes and intercepts of the extrapolated linear portions of each Lineweaver—Burk double-reciprocal plot.

Enzyme source	Cyclic AMP-phosphodiesterase		Cyclic GMP-phosphodiesterase	
	Michaelis constants (μM)	Maximum velocities (nmole/min/mg protein)	Michaelis constants (μM)	Maximum velocities (nmole/min/mg protein)
Larval central nervous system homogenate	10	6.6	8.6	5.8
	21	7.8	19	8.3
34000 × *g* 30 min supernatant	7.5	5.2	—	—
	27	8.6		
0—50% $(NH_4)_2SO_4$ precipitate	8.0	6.1	9.1	5.1
	20	7.6	24.4	9.1
10000 × *g* 20 min (mitochondrial) pellet	6.7	8.0	12	18
	21	16	22	26
Larval brain homogenate	7.7	7.1	7.4	6.6
	20	9.1	19	8.9

In Table V are compiled kinetic constants calculated for a number of enzyme preparations. It clearly emerges that all fractions are kinetically similar, that nerve cord phosphodiesterase exhibits two distinct K_m values for each cyclic nucleotide, and that these constants are relatively close in value (approximately 10 μM and 20 μM). The similarity of the K_m values in all enzyme preparations suggests there may be present a single enzyme in all fractions which has nearly equal affinities for both cyclic nucleotides. In contrast to insect nerve cord crude mammalian cyclic AMP-phosphodiesterase contain two enzymes with well separated apparent K_m values e.g., in rat brain, 4 μM and 120 μM [22]; mouse retina, 0.16 and 2.82 mM [23]; C-6 glial cells, 1.5 μM and 200 μM [24]; rat kidney, 2.7 μM and 95 μM [25] and in human blood platelets, 70 μM and 0.7 mM [26].

In mammalian enzyme preparations it has generally been observed that double-reciprocal plots are non-linear for the hydrolysis of cyclic AMP, but are linear for the hydrolysis of cyclic GMP [22,23,27,28]. Two discrete, micromolar K_m values for the hydrolysis of cyclic GMP by nerve cord phosphodiesterase were found, and this behavior distinguishes the insect preparation from others reported: e.g., 20 μM, rat brain [22]; 0.66 mM, mouse retina [23] and 5.3 μM, cat heart [22].

Effect of pH, imidazole and temperature

Soluble cyclic AMP-phosphodiesterase and GMP-phosphodiesterase activities from the larval central nervous system were maximal at approximately pH 7.5 and were sharply attenuated at higher or lower values of pH. At any value of pH below 7.5, activity was considerably lower in phosphate buffer than in Tris–HCl or imidazole–HCl buffers. The observed pH optimum for nerve cord cyclic AMP-phosphodiesterase is similar to those reported for this enzyme in bovine heart and brain [9,14], frog erythrocytes [29], rat brain [7], uterine smooth muscle [30] and human blood platelets [26], but is somewhat lower than the pH optima (8.5–9.2) reported for fish brain [31], canine heart [32], *M. sexta* larval gut supernatants [33] and cockroach brain [34]. The pH optimum for *M. sexta* central nervous system cyclic GMP-phosphodiesterase is identical to the only other value reported for this enzyme (pH 7.5 for uterine muscle [30]).

At pH 7.5 cyclic AMP-phosphodiesterase was stimulated about 10% in the presence of imidazole as reported for other preparations [7,14,22,32,34,35]. O'Dea et al. [35] reported that imidazole also inhibited the hydrolysis of cyclic GMP.

The dependence of soluble cyclic AMP-phosphodiesterase activity on temperature differed from that of cyclic GMP-phosphodiesterase. Whereas the activity of cyclic GMP-phosphodiesterase was greatest at approximately 35°C and rapidly declined at higher temperatures, the maximal rate of cyclic AMP degradation occurred at about 50°C. Samples of enzyme protein which were pre-incubated at 50°C for 10 min and then assayed at 30°C showed no significant differences between those pre-incubated at 30°C before assay, ruling out the possibility that cyclic GMP-phosphodiesterase activity is selectively denatured at the higher incubation temperature. The dissimilarity of the temperature dependences for the hydrolysis of cyclic AMP and cyclic GMP does not

necessarily support the possibility that separate enzymes exist for the destruction of these two cyclic nucleotides. It is conceivable that a single enzyme having affinity for both nucleotides would, at some temperature, adopt a conformation favoring one substrate.

Effects of bivalent cations

Drummond and Perrot-Yee [15] reported that mammalian cyclic AMP-phosphodiesterase has an absolute requirement for Mg^{2+}. They found that a dialyzed ammonium sulfate fraction from rabbit brain was completely inactive in the absence of Mg^{2+}, that activity could be entirely abolished with 1 mM EDTA and that Mg^{2+} concentrations greater than 0.8 mM were highly inhibitory. These features are not shared by a similarly prepared enzyme from the *M. sexta* larval central nervous system. Low concentrations of Mg^{2+} mildly stimulated the insect phosphodiesterase (50% at 1 mM) above the level of activity observed in the absence of added metals, but high concentrations of Mg^{2+} were not inhibitory. Mn^{2+} and Co^{2+} were moderately more effective in enhancing activity, Ca^{2+} at 1 mM was without effect, but at 10 mM decreased activity by 20%. Zn^{2+}, Cu^{2+} and Fe^{2+} were inhibitory.

These experiments suggest that the insect phosphodiesterase has a weak bivalent metal requirement which could be satisfied in vivo by Mg^{2+}. Therefore it is paradoxical that, upon assaying nerve cord preparations in the presence of 1—10 mM EDTA, activity could not be abolished. Even when enzyme samples were pre-incubated before assay with 30 mM EDTA for 1 h to complex endogenous bivalent metals, greater than 50% of the control cyclic AMP-phosphodiesterase activity remained. These findings are contrary to a number of others where the addition of EDTA abolished activity [15,29,34,36]. The metal requirements of *M. sexta* cyclic GMP-phosphodiesterase were not examined in great detail, but it was established that the effects of Mg^{2+} on its activity were similar to those on cyclic AMP-phosphodiesterase.

Inhibition of phosphodiesterase by miscellaneous compounds

Many compounds, both physiological and non-physiological, inhibit mammalian phosphodiesterase [22,36—40]. Several of these were tested with the intent of finding a potent inhibitor of nerve cord phosphodiesterase that would be useful in a number of cyclic nucleotide-related assays in crude preparations (for example, in adenyl cyclase and protein kinase assays). No agent tested was highly inhibitory when employed at very low concentrations, but some of the compounds investigated were quite inhibitory at the millimolar level (Table VI). None of the compounds specifically inhibited either phosphodiesterase while totally sparing the other. In homogenates, supernatants and ammonium sulfate fractions each cyclic nucleotide inhibited the hydrolysis of the other. With both substrates present at a concentration of 50 μM, the inhibition of cyclic AMP hydrolysis by cyclic GMP (35%) in homogenate samples was similar in magnitude to the inhibition of cyclic GMP hydrolysis by cyclic AMP (27%).

Theophylline, a methylxanthine which has been employed in many systems as an inhibitor of cyclic AMP-phosphodiesterase inhibited both cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase from the insect nerve cord and was more effective than either caffeine or theobromine. A

TABLE VI

INHIBITION OF LARVAL CENTRAL NERVOUS SYSTEM PHOSPHODIESTERASE BY MISCELLANEOUS COMPOUNDS

Enzyme (18 μg of a (0—50) AS fraction prepared as described in Materials and Methods) was preincubated with the stated compound for 10 min at 30°C before assaying for phosphodiesterase in the presence of 50 μM cyclic AMP or cyclic GMP. Assay II was employed for all samples except those with superscript R, these being assayed by the Assay I. Assays were conducted as described in Materials and Methods except that the Mg^{2+} concentration was reduced to 1 mM. Standardized solutions of nucleosides and nucleotides were prepared by measuring absorbance of wavelength of maximum absorbance in neutral solution.

Compund added	Concentration (mM)	Percent inhibition	
		Cyclic AMP-phosphodiesterase	Cyclic GMP-phosphodiesterase
None (control)		0	0
Theobromine	1	34^R	—
Caffeine	1	31^R	—
Theophylline	1	42	66
	5	71	—
	10	89	—
NaF	1	3	0
Alloxan	1	11	12
EDTA	10	54	39
ATP	0.1	44	—
	1	78	—
GTP	0.1	50^R	58^R
	1	100^R	100^R
(Ado-*P*-CH_2-*P*-*P*)	1	39	54
ADP	1	46	60
5′-AMP	0.1	30^R	—
	1	40	57
Adenosine	1	23	49
Guanosine	1	31	51
2′,3′-cyclic AMP	1	46	77
3′,5′-cyclic IMP	1	53	90
PP_i	1	27^R	21^R
	5	53	42
SQ20009	1	46	22
	10^{-2}	23	0
Ro 20-296	1	12	26
	10^{-2}	0	0
Dibutyryl cyclic AMP	$5 \cdot 10^{-2}$	18	10

variety of synthetic compounds far more inhibitory toward mammalian phosphodiesterase than any methylxanthine have been reported [22,37,39,41), and the effects of two of these on nerve cord phosphodiesterase were investigated. R_o 20-2926, which is at least 42 times more potent than theophylline toward rat erythrocyte cyclic AMP-phosphodiesterase [39], was less than one-third as potent as theophylline toward the insect cyclic AMP-phosphodiesterase; it was slightly more effective in inhibiting cyclic GMP-phosphodiesterase. SQ20009, which inhibits rat brain cyclic AMP-phosphodiesterase 50% at a concentration of $2 \cdot 10^{-6}$ M [22], was no more effective than theophylline against the insect cyclic AMP-phosphodiesterase when tested at a concentration of 1 mM. The relative impotency of the mammalian phosphodiesterase inhibitors R_o 20-2926 and SQ20009 toward *M. sexta* central nervous system phosphodiesterase may

be indicative of some fundamental difference in molecular architecture of the insect enzyme.

The effects of modifiers of adenyl cyclase such as alloxan [44], NaF and α,β-methyleneadenosine-5'triphosphate (ado-*P*-Ch_2-*P*-*P*) [42,43] were also tested for their effect on phosphodiesterase. (Ado-*P*-CH_2-*P*-*P*), a competitive, hydrolysis-resistant, substrate for adenyl cyclase [42,43] was an effective inhibitor of nerve cord phosphodiesterase. Alloxan only slightly inhibited nerve cord phosphodiesterase. Sodium fluoride at a 1 mM level was without influence.

Table VI shows that the hydrolysis of cyclic AMP and cyclic GMP was strongly inhibited by ATP and GTP. Inhibition of cyclic AMP-phosphodiesterase by nucleoside triphosphates, has been reported by many workers [7,25,30,36]. The inhibitory effects of ATP and GTP were examined as a function of Mg^{2+} concentration (data not shown), and progressive inhibition of both cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase was observed as Mg^{2+} levels were lowered. GTP was more inhibitory towards cyclic GMP-phosphodiesterase than toward cyclic AMP-phosphodiesterase and more effective than ATP against both enzymes. Nerve cord phosphodiesterase was also inhibited by ADP, 5'-AMP, adenosine and especially by cyclic IMP. Inhibition of activity by 2',3'-cyclic AMP was even greater than that caused by ADP, 5' AMP, or theophylline at equivalent concentrations.

Polyacrylamide disc gel electrophoresis

In view of the small amounts of insect central nervous system tissue available for conventional methods of enzyme purification, it was hoped that polyacrylamide disc gel electrophoresis might provide a simple one-step, many-fold purification using relatively little starting material.

Acrylamide gels were prepared essentially by the technique of Davis [44] and pre-electrophoresed before application of a 34 000 × *g* larval central nervous system supernatant prepared in hypotonic buffer. Following electrophoresis, replicate gels were stained for cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase. Two exceptionally sharp and distinct bands of phosphodiesterase (bands I and II) were observed, corresponding the R_f values of

TABLE VII

PHOSPHODIESTERASE ACTIVITIES IN POLYACRYLAMIDE GEL-SEPARATED BANDS I AND II

Soluble phosphodiesterase from the larval central nervous system was separated by polyacrylamide gel electrophoresis. Two bands of activity, corresponding to R_F values of 0.21 and 0.24, were observed.

Phosphodiesterase band	Units of activity		Ratio cyclic GMP/ cyclic AMP
	Cyclic AMP phosphodiesterase	Cyclic GMP phosphodiesterase	
I	282	547	1.94
II	125	360	2.88
Total	407	907	

0.21 and 0.24. The close adjacency of these bands indicates that the proteins differ only slightly in charge and/or size. Enzyme activities (Assay II) found in the two bands are shown in Table VII. About 23% of the cyclic AMP-phosphodiesterase and 38% of the cyclic GMP-phosphodiesterase activity applied to the gel was recovered in the two bands. Since the relative rates of hydrolysis of cyclic GMP and cyclic AMP by phosphodiesterase in bands I and II differed appreciably, it appears there are at least two discrete enzymes in soluble extracts from the *M. sexta* central nervous system.

Apparent absence of a phosphodiesterase activator in the M. sexta central nervous system

The existence of a protein activator of cyclic AMP-phosphodiesterase has been established in rat, bovine and human brains [9,47,48], in bovine heart [10,49] and in human blood [26]. This activator apparently lacks both tissue and species specificity [9]. The presence of such an activator has not yet been found in any non-mammalian tissue. A distinguishing feature of the protein activator is its exceptional stability to boiling. To investigate whether the insect central nervous system possessed an activator having similar physical properties (i.e., stability at 100°C), a crude "activator fraction" was prepared from 55 larval nerve cords using a procedure similar to that described by Cheung [9]. This "activator fraction", which was totally devoid of phosphodiesterase activity, had no effect on the cyclic AMP-phosphodiesterase, or cyclic GMP-phosphodiesterase activity in a (0—50%) ammonium sulfate fraction, a 105 000 × *g* supernatant, or a polyacrylamide gel-separated fraction. From these experiments one may conclude that a protein activator is not present in the insect central nervous system, was destroyed during boiling, or is in excess or tightly-bound in the preparations examined.

In several mammalian tissues it has been reported that the rate of hydrolysis of cyclic AMP may be accelerated in the presence of low levels of cyclic GMP [1,5] but not at higher concentrations where it begins to inhibit cyclic AMP hydrolysis. In contrast, the rate of hydrolysis of cyclic GMP is decreased at any concentration of cyclic AMP. Experiments were designed to investigate the effect of cyclic GMP on cyclic AMP hydrolysis, and vice versa, in several larval central nervous system preparations. The hydrolysis of 50 μM cyclic AMP (or cyclic GMP) was measured in the presence and absence of 1—10 μM cyclic GMP (or cyclic AMP). In homogenates, ammonium sulfate precipitates and polyacrylamide gel-separated fractions the influence of one cyclic nucleotide on the rate of hydrolysis of the other was purely inhibitory. Therefore, it appears that the cyclic GMP-stimulability of mammalian cyclic AMP-phosphodiesterase is not shared by the insect enzyme system.

Discussion

Phosphodiesterase has been reported to be in great excess over adenyl cyclase in a number of mammalian tissues [14,16,50]. It will be necessary to accurately quantitate adenyl cyclase activity in the *M. sexta* central nervous system to determine if this apparent disparity obtains in the insect. However, if one assumes that nerve cord cyclase activity is comparable in magnitude to that

reported in other insect tissues [51—53], the provisional conclusion may be drawn that phosphodiesterase levels greatly exceed cyclase levels in the insect central nervous system. How, then, may cyclic AMP be expected to accumulate under such apparently catabolic circumstances? It appears likely, as suggested by Cheung [7,36] that phosphodiesterase exists in vivo in a greatly inhibited state.

We have shown that nerve cord phosphodiesterase is effectively inhibited by millimolar levels of ATP and PP_i, the substrate and product, respectively, of adenyl cyclase. Although the intracellular concentrations of ATP and PP_i in the *M. sexta* central nervous system are not known, Cheung [36] has estimated that levels of ATP in rat brain may reach 4 mM. This concentration of ATP, particularly in conjunction with low intracellular levels of Mg^{2+}, would severely inhibit both cyclic AMP-phosphodiesterase and cyclic GMP-phosphodiesterase in the nerve cord. Circumstances favorable to the formation of cyclic AMP, i.e., activated adenyl cyclase in the presence of moderate levels of ATP, would presumably also lead to suppression of phosphodiesterase activity (through inhibition by ATP, PP_i and also 5′-AMP). Thus it appears that a transient elevation of cyclic AMP levels in the presence of a great excess of phosphodiesterase is in part due to the susceptibility of phosphodiesterase inhibition by a variety of endogenous substances. Of these, nucleoside triphosphates would appear to be the most potent, although numerous compounds (PP_i, ADP, AMP, adenosine, etc.) may participate in the general framework of control mechanisms.

Kinetic analyses of various fractions from the *M. sexta* larval nerve cord disclosed two Michaelis constants for the hydrolysis of cyclic AMP, and two for cyclic GMP. These constants did not differ appreciably in soluble and particulate preparations, ammonium sulfate and subcellular fractions and brain homogenates. Biphasic kinetic behavior observed (Fig. 1, Table V) may be indicative of multiple enzymes with disparate affinities for cyclic nucleotide, and downward curvature in double-reciprocal plots may simply reflect a summation of velocities. Alternatively, it may be due to a single homotrophic, cooperative regulatory enzyme, i.e., one which contains two or more binding sites for substrate and binding of the first substrate to one of these sites affects subsequent binding to the other. If there is present in our preparations a single enzyme (or isozymes having similar substrate affinities), the experimental data only allow the generalization that, kinetically, it apparently contains multiple catalytic sites or a single site which is susceptible to substrate activation.

Cyclic AMP- or cyclic GMP-phosphodiesterase activities could not be distinguished by their metal requirements, pH optima, or subcellular distribution, nor was there a pronounced differential susceptibility of the two activities to a variety of inhibitors. Our findings support the idea that the degradation of both cyclic AMP and cyclic GMP in the insect nerve cord may result from the action of a single enzyme or kinetically similar enzymes with nearly equal affinities for both cyclic nucleotides. This conclusion is advanced with caution as it is possible that some forms of phosphodiesterase present in small quantities were masked. The existence in the insect central nervous system of cyclic AMP-specific or cyclic GMP-specific phosphodiesterase which have been reported in some mammalian systems, cannot be disallowed.

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