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CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN THE CRICKET, *ACHETA DOMESTICUS*

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Summary

Exceptionally high levels of guanosine 3',5'-cyclic monophosphate (cyclic GMP) in the accessory reproductive gland of the male house cricket, *Acheta domesticus*, led to an investigation of cyclic nucleotide phosphodiesterase (EC 3.1.4.—) as a possible regulatory enzyme. Cricket cyclic nucleotide phosphodiesterase activity with cyclic GMP or cyclic AMP as substrate had a pH optimum around 9.0, required Mg^{2+} or Mn^{2+} for maximal activity, and was inhibited by EDTA and methylxanthines. Cyclic GMP phosphodiesterase occurred mainly in the soluble fraction of homogenates of accessory glands or whole crickets, but cyclic AMP phosphodiesterase in the accessory gland was primarily particulate. Kinetic analysis indicated three forms of cyclic GMP phosphodiesterase, with K_m values at 2.9 μM, 71 μM and 1.5 mM. Chromatography of whole cricket or accessory gland extracts on DEAE cellulose gave an initial peak having comparable activity with either cyclic GMP or cyclic AMP, and a second peak specific for cyclic AMP.

There were no appreciable changes in the specific activity or kinetic properties of accessory gland cyclic GMP phosphodiesterase during a developmental period over which cyclic GMP levels rise more than 500-fold. Thus, the accumulation of cyclic GMP in the accessory gland is probably not associated with concomitant developmental modulation of phosphodiesterase activity.

Introduction

The accumulation of exceptionally high levels of cyclic GMP in the male accessory gland of the house cricket, *Acheta domesticus*, provides an interest-

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Abbreviations are: cyclic GMP, guanosine 3',5'-cyclic monophosphate; cyclic AMP, adenosine 3',5'-cyclic monophosphate.

ing system for investigating the regulation and function of cyclic GMP. During two weeks after the cricket molts to the adult stage, cyclic GMP in the accessory gland rises from virtually undetectable levels to over 1500 pmol/gland, an increase of more than 500-fold [1]. The content in the mature gland is about 100 μmol/kg, which is 100 to 1000-fold higher than that in most animal tissues.

The functional relationship between cyclic nucleotides and their phosphodiesterases (EC 3.1.4.—) has received considerable attention, and several modes of regulation have been proposed. These include induction of de novo synthesis of phosphodiesterase by increased intracellular cyclic AMP [2], and interaction with a Ca^{2+}-dependent protein activator [3], which apparently occurs in most animal species [4]. Negative modulation by a low molecular weight phosphodiesterase inhibitor, isolated from rat adipocytes, has also been described [5].

The association of elevated levels of cyclic AMP [6] and cyclic GMP [7] with reduced phosphodiesterase activity is particularly intriguing. The pattern of accumulation of cyclic GMP in the mutant mouse retina [7] resembles that in the cricket accessory gland, and suggests the importance of examining phosphodiesterase as a possible regulator of accessory gland cyclic GMP. Properties of cricket cyclic GMP and cyclic AMP phosphodiesterases and their resolution into discrete activities by DEAE cellulose chromatography are the subjects of this report.

Materials and Methods

Insects

The house cricket, *Acheta domesticus*, was reared in the laboratory as described previously [1]. Dissections were at room temperature in a cricket Ringers containing 150 mM NaCl, 8 mM KCl, 6 mM $MgSO_4$, 4.4 mM $CaCl_2$, and 15 mM *N*-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) adjusted to pH 7.5 with NaOH. Male crickets were used for all assays.

Reagents

Tritiated cyclic nucleotides (cyclic [^{3}H]AMP, 27 Ci/mmol; cyclic [^{3}H]GMP, 19 Ci/mmol) were from Amersham/Searle and ^{14}C-labeled nucleosides ([^{14}C]-guanosine, 49.2 Ci/mol; [^{14}C]adenosine, 51.2 Ci/mol) were from New England Nuclear. Unlabeled cyclic GMP was from Nutritional Biochemical Corp., and 5′-nucleotidase (*Crotalus atrox* venom), alumina (Basic Type WB-2 and Neutral Type WN-3), and caffeine were from Sigma. Theophylline was from Calbiochem, and 3-isobutyl-1-methylxanthine was from Aldrich.

Preparation of extracts

Tissue extracts were prepared in 45 mM triethanolamine/Cl, pH 8.0, at 0°C using a glass homogenizer with a motor-driven teflon pestle. Accessory glands were generally used at 2—5 per ml, and whole crickets, after removal of wings and legs, at 1 per 5 ml. Extracts made from whole crickets (1 per ml) for DEAE chromatography were too concentrated for adequate disruption by this method, and mechanical homogenization was followed by disruption with a Brink-

mann Polytron, using two 30-s pulses at full speed, with 2 min between pulses. Similar treatment was applied to extracts used for testing the effects of ions and inhibitors. Accessory gland extracts were not disrupted with the Polytron because they foamed excessively and lost considerable activity. Extracts were generally dialyzed against 100 volumes of the same buffer for 8—10 h, with 1—3 changes, depending on the necessity for complete removal of endogenous cyclic GMP or metal ions. Extracts for DEAE chromatography and for determination of subcellular distribution were not dialyzed.

Determination of protein

Accessory glands contain a significant amount of stored secretory protein which is for the most part insoluble in aqueous buffer. Since the amount of this protein increases during development [1], specific activities based on total protein would not provide a good basis for comparison of developmental changes in tissue phosphodiesterase activity. Specific activities were therefore based on protein in the supernatant fraction after 10 min centrifugation at 8000 × *g*. Protein was determined by the method of Bramhall et al. [8], using Xylene Brilliant Cyanin G (Esbe Laboratory Supplies, Toronto, Canada) as the stain and bovine serum albumin as the standard.

Subcellular distribution

To determine the distribution of phosphodiesterase in accessory glands, extracts were centrifuged at 1000 × *g* for 10 min, then at 16 000 × *g* for 15 min and at 120 000 × *g* for 1 h. The two low-speed pellets were resuspended in the initial volume of buffer and washed twice; the 120 000 × *g* pellet was washed only once. To avoid dilution of the enzyme, the washings were not added to the original supernatants. Activity in extracts from whole crickets was determined after centrifugation at 1000 × *g* for 10 min and at 120 000 × *g* for 1 h.

Chromatography on DEAE cellulose

Enzyme from rat liver was prepared exactly as described by Russell et al. [9], except that the homogenate was treated with a Brinkmann Polytron instead of a sonicator. The filtered supernatant was chromatographed on a column of Whatman DE-32 equilibrated with 50 mM Tris/acetate, pH 6.0, containing 3.75 mM 2-mercaptoethanol, and eluted with a gradient of sodium acetate as described [9]. Fractions were assayed directly at 50 μM substrate. For direct comparison of cricket phosphodiesterase with rat liver enzyme, 11 adult male crickets were homogenized in 12 ml distilled water and disrupted with the Polytron. After centrifugation at 8000 × *g* for 15 min, the filtered supernatant (10.5 ml) was chromatographed as described above.

Since recovery of cricket phosphodiesterase activity from columns run at pH 6.0 was low, more extensive comparisons between phosphodiesterase from whole crickets and from accessory glands were made at pH 8.0. Crickets (10 adult males, from which accessory glands had been removed) or accessory glands (130 glands, dissected from adult males, quickly frozen on dry ice, and stored at −20°C until use) were homogenized in 45 mM triethanolamine/Cl pH 8.0, and disrupted with the Polytron (whole crickets only) by two 30-s pulses at full speed with 2 min between pulses. The suspension was centrifuged at

1000 × *g* for 10 min, the pellet was washed with 5 ml of buffer and the combined supernatants (20 ml) were filtered and applied to a DE-32 column equilibrated with 45 mM triethanolamine/Cl, pH 8.0. The column was washed with about 200 ml of the same buffer, and activity was eluted with a 0.05 to 0.5 M linear NaCl gradient in a total volume of 600 ml. Fractions were assayed directly at 50 or 500 μM substrate.

Purification of substrates

Tritiated cyclic nucleotides contained unidentified contaminants which co-chromatographed with the respective nucleosides on alumina columns, giving high assay blanks. To remove contaminants, cyclic nucleotides were purified on alumina columns [10]. Peak fractions from the alumina chromatography were pooled and applied directly to a 0.7 × 3 cm Dowex column (AG 1 x8, 200—400 mesh, formate form, equilibrated with distilled water) and washed with 10 ml of distilled water. Cyclic AMP was eluted with 14 ml of 2 M formic acid, and cyclic GMP was washed with the latter and eluted with 18 ml of 4 M formic acid. Effluents were lyophilized, dissolved in distilled water and stored at −20°C. Such preparations remained satisfactory for use for about 2 months.

Phosphodiesterase assays

Phosphodiesterase was assayed according to the method of Filburn and Karn [11] in a final volume of 0.15 ml containing 75 mM triethanolamine/Cl, pH 8.0, 5 mM or 10 mM (in kinetic studies) $MgSO_4$, cyclic nucleotide (40 000 cpm/tube at 5—100 μM substrate; 300 000 cpm/tube at 0.2—5 mM substrate), 0.2 unit of 5′-nucleotidase, and appropriate amounts of enzyme. After incubation at 30°C, usually for 30 min, reactions were terminated by addition of 30 μl of 1 M acetic acid containing about 3000 cpm of the appropriate ^{14}C-labeled nucleoside to monitor recovery during chromatography. Tritiated nucleosides were separated from substrate on aluminum oxide columns (1 g basic alumina for cyclic GMP; 1.5 g neutral alumina for cAMP) pre-equilibrated with 25 ml 0.1 M ammonium acetate buffer, pH 4.0. Nucleosides were eluted with 2 ml of the same buffer, collected in scintillation vials, and counted with 15 ml of Triton X-100 scintillator. Recovery from the columns was 60—70%, and all values were normalized to 100% recovery.

In some cases, a two step reaction was used, in which the 5′-nucleotidase was omitted from the reaction mixture. Phosphodiesterase activity was then terminated by heating for 75 s in a boiling water bath. After cooling on ice, the 5′-nucleotidase was added and the reaction continued for an additional 25 min at 30°C before stopping with acetic acid. In no case were we able to detect a difference between the one and two-step reactions, and the one-step assay was used unless otherwise indicated.

The two-step procedure was modified to test the effects of various cations and inhibitors on phosphodiesterase activity. In this case 15 μl containing 0.2 unit of 5′-nucleotidase and 2.5 μmol $MgSO_4$ was added to the boiled tubes, and the reaction was continued for 30 min at 30°C. The heavy metals Cu^{2+} and Zn^{2+} inhibited nucleotidase activity even in the presence of excess Mg^{2+}, and

this inhibition was eliminated by addition of 5 μmol of EDTA prior to addition of 5′-nucleotidase.

Assay blanks, containing buffer instead of enzyme, boiled enzyme, or enzyme plus acetic acid gave the same result, and generally only buffer blanks were used. All assays were in duplicate, and were linear with time and enzyme concentration. For kinetic analyses, time and/or enzyme concentration were varied so as to keep hydrolysis to less than 25% of the substrate.

Results

General characteristics

Phosphodiesterase activity from either accessory glands or whole crickets had similar properties with cyclic GMP or cyclic AMP as substrate. Reaction rates were linear with time and enzyme concentration for at least 90 min, or until 50—60% of the substrate had been hydrolyzed. Like other cyclic nucleotide phosphodiesterases, the cricket enzyme, assayed at 50 μM substrate, had an alkaline pH optimum, with maximal activity at pH 8.5—9.0 for cyclic GMP and 9.0—9.5 for cyclic AMP. Assays were carried out at pH 8.0, where activity was about 80% of maximum. Snake venom 5′-nucleotidase did not activate crude phosphodiesterase from accessory glands or whole crickets or rat liver enzyme after partial purification by DEAE chromatography. Such activation has been reported for more extensively purified phosphodiesterase from bovine brain [12,13].

Unlike many vertebrate phosphodiesterases, the enzyme from crickets was not stable during prolonged storage at 4°C, and about 20% of cyclic GMP and 10% of cyclic AMP activity were lost by 24 h. Activity assayed at high substrate concentrations diminished less rapidly than at low substrate. Enzyme was generally used within 12 h of preparation, and fractions from DEAE chromatography were assayed within 24 h. Attempts to stabilize the activity with 0.1 or 2 mM dithiothreitol or 50 mM NaCl added to the homogenization buffer were not successful.

Effects of metal ions and inhibitors

Cricket cyclic GMP phosphodiesterase was activated by Mg^{2+} at concentrations up to 100 mM (Fig. 1). Partial activity was observed in the absence of added Mg^{2+}, the proportion of this activity varying slightly with cyclic GMP concentration. Cyclic AMP phosphodiesterase had less activity in the absence of added metal ions (Table I). With both substrates, the chelating agent EDTA was highly inhibitory, indicating that the apparently Mg-independent activity was probably due to tightly bound metal not removed during dialysis. In contrast, EGTA was considerably less effective in inhibiting Mg-independent activity (Table I). The peak of cyclic AMP enzyme resulting from DEAE chromatography of whole cricket extracts showed maximum activity at 25—50 mM Mg^{2+}, whereas the cyclic GMP peak plateaued by 5—10 mM Mg^{2+} with substrate concentrations of 50 μM to 5 mM cyclic nucleotide. The level of Mg^{2+} required for maximum activity increased with increasing substrate concentrations.

With the exception of Mn^{2+}, which activated cyclic AMP and, to a lesser extent, cyclic GMP phosphodiesterase more strongly than Mg^{2+}, other divalent

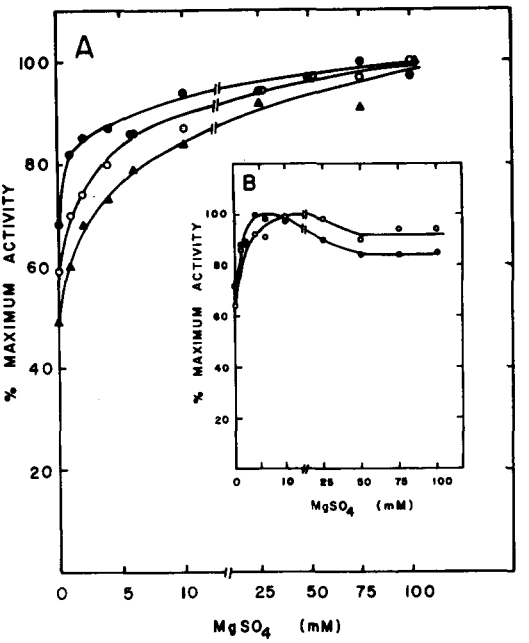

Fig. 1. Effect of Mg^{2+} on cricket cyclic GMP phosphodiesterase activity. Filtered and dialyzed extract from whole crickets from which the accessory gland had been removed (A), or dialyzed extract from accessory glands (B) was assayed at 50 μM (●), 0.5 mM (○), and 5 mM (▲) cyclic GMP, in the presence of various concentrations of Mg^{2+}. Accessory gland values are averaged from 2 independent experiments in which the variation was generally less than 5%.

TABLE I

EFFECT OF METAL IONS AND INHIBITORS ON CRICKET PHOSPHODIESTERASES

Phosphodiesterase activity was measured by the two-step method with 50 μM cyclic nucleotide and various additions at pH 8.0. A dialyzed 8000 × *g* supernatant of extract from whole crickets was used as the source of enzyme and cations were from chloride or sulfate salts. After termination of the phosphodiesterase reaction, EDTA was added to tubes containing Zn^{2+} and Cu^{2+} to prevent inhibition of the nucleotidase. Values are averages of two independent determinations, which generally agreed within 5%.

Addition (mM)	Activity relative to that found with 10 mM Mg^{2+} (%)							
	Cyclic GMP				Cyclic AMP			
	0	0.5	3	10	0	0.5	3	10
None	83				28			
Mg^{2+}		91	96	100		80	95	100
Mn^{2+}		106	110	102		137	126	118
Ca^{2+}		76	46	32		22	22	18
Cu^{2+}		10	<2	<2		<2	<2	<2
Zn^{2+}		42	9	8		6	<2	<2
Mg^{2+} (3 mM) + Ca^{2+}		98	93	—*		104	82	—
EDTA		10	6	8		<2	<2	2
EGTA		80	72	54		26	17	10
Caffeine		72	52	30		32	22	14
Theophylline		78	54	28		30	18	10
3-isobutyl-1-methylxanthine		26	8	—		13	5	—

* Not determined

cations were inhibitory at the concentrations tested (Table I). Tests with $MnSO_4$ and $MnCl_2$ indicated that the anions had no effect on enzyme activity. The inhibitors caffeine and theophylline gave a 50—65% decrease in activity at 10 mM, while 3-isobutyl-1-methylxanthine was significantly more effective.

Subcellular distribution

Cyclic GMP and cyclic AMP phosphodiesterase activities differed markedly in subcellular distribution in the accessory gland, for cyclic GMP activity was principally soluble, while most of the cyclic AMP activity was particulate at both 50 μM and 1 mM substrate (Table II). In extracts from whole crickets, activity for both substrates was mostly soluble. No significant phosphodiesterase activity was detected in mitochondrial or microsomal pellets.

Distribution in the body and changes during development

Phosphodiesterase activity in the various body regions of the cricket ranged from 280—1200 pmol/min per mg soluble protein for cyclic GMP and from about 900—6000 pmol/min per mg soluble protein for cyclic AMP at 50 μM substrate (Table III). In all cases, cyclic AMP was the preferred substrate, but there was significant variation in the ratio of cyclic AMP/cyclic GMP activity. Activities in the accessory gland were comparable to those in other regions of the body. Moreover, there was little change in phosphodiesterase activity in the accessory gland during 11 days after the final molt (Table IV). The seminal vesicles, which adjoin the accessory gland and are not easily removed by dissection, contribute about 8% of the cyclic GMP phosphodiesterase activity measured in accessory glands at 50 μM substrate.

Kinetic complexity and multiple forms of phosphodiesterase

Phosphodiesterases from many sources exhibit kinetic complexity as indicat-

TABLE II

SUBCELLULAR DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

Accessory glands or whole crickets were homogenized in 45 mM triethanolamine/Cl, pH 8.0, and centrifuged at the indicated *g* values. Pellets were washed twice by resuspension and centrifugation, and the washings were discarded. Values are means and ranges of two separate determinations. Results from the accessory gland are expressed as percentage of total recovered activity (89% for cyclic GMP and 78% for cyclic AMP), and results for whole crickets are expressed as percentage of activity in the unfractionated homogenate. Mechanical homogenization of accessory glands in a glass-teflon or in a ground glass homogenizer did not alter the distribution of activity.

Fraction	Cyclic GMP		Cyclic AMP	
	50 μM	1 mM	50 μM	1 mM
Accessory gland				
1 000 × *g* pellet	24 ± 3	36 ± 2	80 ± 2	78 ± 4
16 000 × *g* pellet	1	2	2	2
120 000 × *g* pellet	<1	2	<1	<1
120 000 × *g* supernatant	73 ± 2	60 ± 4	17 ± 2	20 ± 4
Whole cricket				
1 000 × *g* supernatant	84 ± 2		76 ± 5	
120 000 × *g* supernatant	81 ± 4		76 ± 0	

TABLE III

DISTRIBUTION OF PHOSPHODIESTERASE ACTIVITY IN THE CRICKET

Tissues from adult male crickets were homogenized in 45 mM triethanolamine/Cl, pH 8.0, and dialyzed against the same buffer. Assays were at 50 μM substrate, and results are averages of 2 or 3 independent determinations.

Source	Activity, pmol/min per mg soluble protein		
	cyclic GMP	cyclic AMP	Ratio cyclic AMP/cyclic GMP
Head	1150	5720	5.0
Thorax	280	1830	6.5
Accessory gland	620	2300	3.7
Testes	480	900	1.9
Rest of abdomen	1080	1620	1.5

ed by non-linear Lineweaver-Burk plots. In several cases, this has been shown to result from the activities of multiple enzymes with different affinities for substrate. Kinetic analysis revealed similar complexity in phosphodiesterase from the cricket. Millimolar levels of substrate were required to approach saturation, and Lineweaver-Burk plots of accessory gland preparations indicated three kinetically distinct forms of cyclic GMP phosphodiesterase (Fig. 2; Table V). Assays on glands dissected at 0—32 h, 4—6 days, and 10—14 days after the final molt indicated no major change in K_m and V values during maturation of the gland (Table V). The possibility of a slight upward trend in intermediate K_m values, however, is intriguing, for intracellular levels of cyclic GMP may be in the micromolar range [1]. The higher V values obtained with accessory glands from crickets of random ages may be due to the fact that these glands

TABLE IV

CYCLIC GMP PHOSPHODIESTERASE ACTIVITY IN THE MALE CRICKET ACCESSORY GLAND DURING TIME AFTER THE FINAL MOLT

Accessory glands from 2 crickets of each age were homogenized in 1.0 ml of 45 mM triethanolamine/Cl, pH 0.8, and dialyzed against the same buffer. Assays were with 5 mM Mg^{2+} and the indicated substrate concentrations. Assays with 50 μM cyclic GMP were done 8 h after homogenization and assays with 2 mM cyclic GMP after storing the enzyme at 4°C overnight.

Age (h)	Cyclic GMP phosphodiesterase activity			
	pmol/min per gland		pmol/min per mg soluble protein	
	50 μM	2 mM	50 μM	2 mM
4	214	351	738	1210
20	204	377	638	1178
31	290	523	547	987
72	396	693	542	949
93	304	584	475	912
104	336	618	498	920
143	352	644	424	776
168	372	647	510	886
217	494	797	617	992
260	356	617	445	771

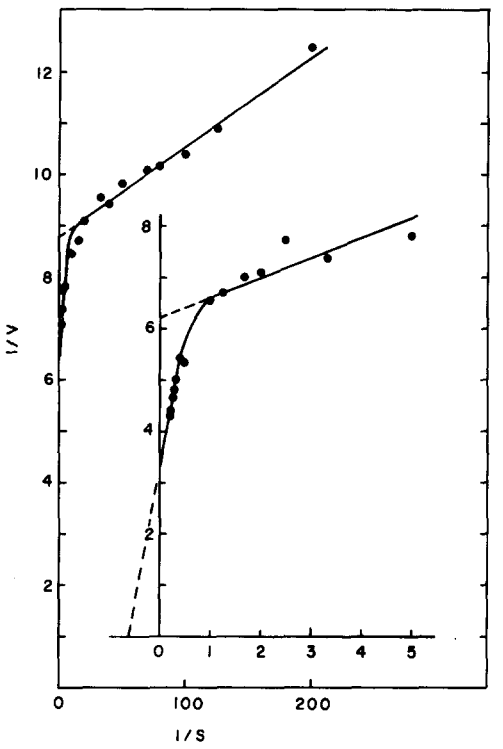

Fig. 2. Lineweaver-Burk plot of accessory gland cyclic GMP phosphodiesterase at 5—5000 μM substrate. Dialyzed extract from 26 accessory glands (Expt. I of Table V) was assayed in the presence of 10 mM Mg^{2+} and various concentrations of cyclic GMP. Inset: expansion of $1/s = 0 - 5$. Abscissa: $(mM)^{-1}$; ordinate: $(pmol/30\ min)^{-1} \cdot 10^5$.

TABLE V

KINETIC CHARACTERISTICS OF ACCESSORY GLAND CYCLIC GMP PHOSPHODIESTERASES

Glands were dissected from adult crickets of the indicated ages after the final molt and stored frozen until about 25 had been collected. Glands from crickets of mixed age were used immediately after dissection. Homogenization was in 45 mM triethanolamine/Cl, pH 8.0, at 5 glands per ml, and assays were in the presence of 10 mM Mg^{2+}. Values were determined graphically, using straight lines fitted by eye (see Fig. 2), without correction for the influence of each enzyme on the kinetics of the others. Units for V are pmol/min per gland.

Age	Low K_m		Intermediate K_m		High K_m	
	K_m (μM)	V	K_m (μM)	V	K_m (mM)	V
0—32 hours	4.3	202	28	386	1.0	722
4—6 days	3.1	328	26	444	1.4	1008
10—14 days	2.2	389	154	652	1.1	1042
Mixed						
Expt. I	2.0	733	65	1030	1.7	1890
Expt. II	2.7	787	84	1133	2.2	2380
Average ± S.E. mean (n = 5)	2.9 ± 0.4		71 ± 23		1.5 ± 0.2	

were freshly dissected whereas those for the developmental series were stored at −20°C prior to use. Extracts from whole crickets from which the accessory glands had been removed had similar kinetics, with K_m values at 4.7 μM, 130 μM, and 2.2 mM (averaged from two experiments which agreed to within 5%).

It was of interest to determine whether these kinetic forms could be separated by DEAE-cellulose chromatography, which has been used for partial purification of phosphodiesterases from other sources. To provide a basis for comparison of the cricket enzymes, we chromatographed rat liver phosphodiester-

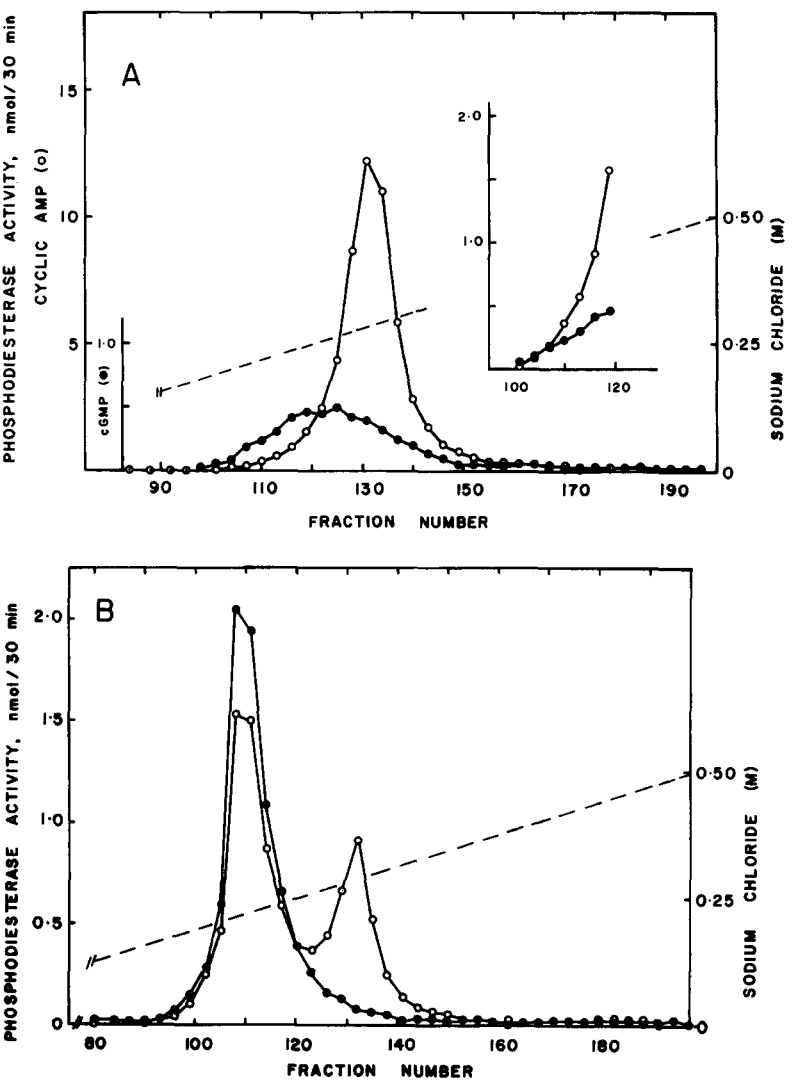

Fig. 3. DEAE chromatography of phosphodiesterase from (A) whole crickets from which accessory glands had been removed and (B) accessory glands at pH 8.0. Activity was eluted with a linear 0.05—0.5 M gradient of NaCl in 45 mM triethanolamine/Cl, pH 8.0, in a total volume of 600 ml. The column (1.7 × 30 cm) was pumped at 45 ml/h, and 4 ml fractions were collected. Fractions were assayed directly at 50 μM cyclic GMP (•) and 50 μM cyclic AMP (o). The inset (A) shows activities of both substrates plotted on the same scale.

ase according to the methods of Russell et al. [9]. All the activity applied to the column was recovered, and it was resolved into three peaks which had, in order of elution, greater activity with cyclic GMP (I), similar activity with both cyclic nucleotides (II), and greater activity with cyclic AMP (III), as described previously [9]. Chromatography of whole cricket extracts under identical conditions gave a small shoulder with approximately equal activity for both cyclic nucleotide substrates preceding the majority of the activity, which was specific for cyclic AMP. 2 discrete peaks of cAMP activity occurred consistently, suggesting two forms of the enzyme(s) under these conditions. Unlike rat liver phosphodiesterase, however, activity in cricket extracts was quite unstable during this procedure, and the highest recoveries we have been able to obtain from DEAE chromatography at pH 6.0 were 10% for cyclic GMP and 15% for cyclic AMP. With either rat liver or cricket extracts, no phosphodiesterase activity eluted before application of the salt gradient.

Better recovery of cricket phosphodiesterase activity was obtained when columns were run at pH 8, but the enzyme continued to lose activity during fractionation. Extracts from whole crickets from which the accessory glands had been removed showed a major peak of cyclic AMP activity which obscured a smaller peak of cyclic GMP phosphodiesterase (Fig. 3A). Recoveries were 40% for cyclic GMP, and 70% for cyclic AMP. Similar profiles were obtained when fractions were assayed with 50 μM or 0.50 mM substrate.

In the accessory gland, cyclic AMP activity was largely insoluble, permitting better resolution of the predominantly soluble cyclic GMP activity (Fig. 3B). About 65% of the activity for each nucleotide was recovered, and two discrete peaks of activity were resolved. The first (I) eluted at 0.22 M NaCl and had comparable activity for either nucleotide; the second (II) was specific for cyclic AMP, and eluted at 0.28 M NaCl, as did the cyclic AMP peak from whole crickets. These characteristics suggest that peaks I and II from crickets correspond to peaks II and III from rat liver, respectively. A preliminary experiment indicated that the concentrated enzyme from accessory gland peak I had K_m values for cyclic GMP at 6 and 30 μM, but the high K_m value observed in unfractionated preparations appeared to be missing. Thus it is possible that the high K_m enzyme represents a species of cyclic AMP phosphodiesterase hydrolyzing cyclic GMP with low affinity.

Discussion

The conditions for activity of cricket cyclic nucleotide phosphodiesterases are similar to those of other vertebrate and insect phosphodiesterases. Hydrolysis of both cyclic AMP and cyclic GMP has an alkaline pH optimum, and Mg^{2+} or Mn^{2+} are required for maximal activity. About 80% of cyclic GMP activity and 30% of cyclic AMP activity were independent of added metal ion, but inhibition by EDTA indicated that this was probably due to bound ions not removed by dialysis (cf. ref. 14). Phosphodiesterases not inhibited by EDTA, however, have been detected in other insect tissues [10,15,16]. Phosphodiesterase from crickets is inhibited by methylxanthines to an extent comparable to vertebrate enzymes [17], 3-isobutyl-1-methylxanthine being a more effective inhibitor than caffeine or theophylline. Unlike some vertebrate enzymes,

cricket phosphodiesterase was not stable to prolonged storage at 4°C, and dithiothreitol did not seem to improve stability. As with silkworm phosphodiesterase [18], activity for cyclic GMP was more labile than that for cyclic AMP.

Cyclic GMP phosphodiesterase from both the accessory gland and whole crickets was mostly soluble, whereas cyclic AMP activity was mostly soluble in whole crickets, but mainly particulate in the accessory gland. There was no appreciable difference in distribution of activity assayed at 50 μM or 1 mM of either substrate, although different subcellular distributions of low and high K_m activities from other sources have been reported [19].

Kinetic analysis of cyclic GMP phosphodiesterase from accessory glands or from whole crickets gave triphasic Lineweaver-Burk plots, with K_m values around 3 μM, 71 μM, and 1.5 mM. K_m values reported for cyclic GMP phosphodiesterase from a variety of vertebrate sources range from 2 to 180 μM [19], and it is possible that enzymes with higher K_m values have escaped detection, since millimolar concentrations of cyclic GMP are not often used in isotopic assays. For cyclic GMP phosphodiesterase from whole silkworm larvae (*Bombyx mori*) a single K_m of 2 μM was reported [20], but activity was not measured at substrate levels above 10 μM. Larval fat body from the silkmoth *Hyalophora cecropia* showed cyclic GMP phosphodiesterase activity with K_m values of 2 μM and 125 μM [10]. Cyclic AMP phosphodiesterase from whole silkworms gave triphasic Lineweaver-Burk plots, but the low-affinity enzyme, which hydrolyzed both 2′,3′- and 3′,5′-cyclic nucleotides, had distinct chromatographic properties on DEAE cellulose [18]. Chromatographic analysis of cricket extracts failed to resolve such an enzyme.

DEAE chromatography of vertebrate phosphodiesterases typically gives three distinct peaks of activity: a cyclic GMP-favoring component, a second peak with comparable activity for both cyclic nucleotides, and a third peak with specificity for cyclic AMP [21]. We have reproduced these findings with rat liver extract. Under the conditions used for rat liver, cricket phosphodiesterase gave quite different results. Most of the activity from whole crickets preferred cyclic AMP as substrate, but a small shoulder of activity hydrolyzed both substrates at similar rates. Chromatography at a higher pH with substantially better recovery gave essentially the same result. In accessory glands, most of the cyclic AMP phosphodiesterase was insoluble, and the soluble cyclic GMP and cyclic AMP activities were better resolved. However, in neither preparation was there evidence for an enzyme with distinct specificity for cyclic GMP.

These studies do not eliminate the possibility of a highly labile cyclic GMP-specific enzyme, since even under the best conditions recovery of activity was only 65%. Moreover, we were not able to resolve the cyclic GMP activity into distinct peaks corresponding to each of the activities indicated by kinetic analysis. However, this might be achieved by other chromatographic techniques, which have been used to separate cyclic GMP phosphodiesterases from other insects [10,20].

In the retina of the mutant mouse C3H/HeJ (rd), an accumulation of cyclic GMP to levels comparable to those in the cricket accessory gland is associated with the absence of a high-K_m phosphodiesterase [7]. Thus it seemed worth asking whether phosphodiesterase may play a similar regulatory role in the

cricket gland. In newly molted adult male crickets, levels of cyclic GMP in the accessory gland begin to increase steeply after an initial lag of about 2 days [1]. During this time however, there were no corresponding changes in the activity or the kinetic characteristics of accessory gland cyclic GMP phosphodiesterase. Thus there was no indication of regulation of cyclic GMP levels by phosphodiesterase. However, our analysis cannot exclude the possibility that phosphodiesterase activity measured in the entire gland is not representative of that in each of the various types of secretory tubule, only certain of which may be responsible for the accumulation of cyclic GMP. If the results do reflect the situation in the cyclic GMP-producing cells there is adequate activity to hydrolyze all the accumulated cyclic GMP in a few minutes, and cyclic GMP would need to be protected from the enzyme. One of the protein products of the accessory gland or an endogenous phosphodiesterase inhibitor might play such a role (cf. refs. 22 and 5).

The function of the accessory gland is to produce spermatophores, proteinaceous capsules used to transfer sperm to the female in copulation. The occurrence of cyclic GMP in spermatophores of both normal [1] and castrated crickets (our unpublished data) indicates that spermatophore cyclic GMP derives from the accessory gland, and not from the sperm. The amount of cyclic GMP lost daily in spermatophore formation (cf. ref.23) is comparable to the daily accumulation of cyclic GMP in the gland prior to the beginning of spermatophore formation (3—6 days after the final molt), when levels are increasing most rapidly [1]. Thus it is conceivable that accessory gland cyclic GMP is synthesized primarily for export, and is stored extracellularly in the lumina of accessory gland tubules, where it would be inaccessible to cyclic GMP phosphodiesterase. Further work is in progress to test this possibility.

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