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CYCLIC NUCLEOTIDE-STIMULABLE PROTEIN KINASES IN THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA*

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Summary

Cyclic nucleotide-stimulable protein kinase (EC 2.7.1.37) has been studied in crude extracts from the central nervous system of the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae). The insect kinase was sulfhydryl-sensitive and required Mg^{2+} for optimal activity. Polyacrylamide gel electrophoresis of supernatants demonstrated the presence of multiple kinases in the larval nerve cord. At low concentrations, cyclic AMP was a much more potent activator of soluble and particulate activities than was cyclic GMP. The specific activity of soluble kinase and the magnitude of its activations by cyclic AMP were greater in the adult than in the larval central nervous system. The exogenous protein substrate specificity of the insect enzyme was similar to that of rat brain kinase with the sole exception that protamine was more readily phosphorylated than histone by nerve cord kinase. It was observed that cyclic AMP lowered the K_m of *Manduca sexta* kinase for ATP, a phenomenon which is apparently nervous tissue-specific in mammals. An effective inhibitor of cyclic AMP-dependent protein kinase was prepared from the larval central nervous system.

Introduction

Protein kinases (EC 2.7.1.37) which are activated by low levels of cyclic AMP and utilize ATP as a phosphate donor, are capable of phosphorylating a wide variety of particulate and soluble proteins [1,2]. These enzymes have been studied in a great number of tissues and phyla [3,4] but have not been

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characterized in insect nervous tissue. Current evidence supports the view that cyclic AMP-dependent protein kinases are allosteric enzymes [5,6] and that cyclic AMP promotes the dissociation of the holoenzyme into a catalytic subunit (cyclic AMP-independent kinase) and a cyclic AMP-regulatory subunit complex. It has been postulated that all the diverse effects of cyclic AMP are mediated through the regulation by cyclic AMP of the activity of protein kinases [3,7].

Protein kinases which are activated by cyclic GMP have also been described [4,6,8] and found to be particularly abundant in arthropods [4] but not in mammalian tissues [9]. Miyamoto et al. [6] demonstrated that cyclic GMP-dependent protein kinases behave in a fashion similar to most cyclic AMP-dependent protein kinases with respect to activation and dissociation into regulatory and catalytic subunits. Kuo et al. [4] found that in many tissues of Lepidoptera cyclic GMP-dependent protein kinases were relatively more abundant than cyclic AMP-dependent kinases and advanced the idea that in certain insect tissues cyclic GMP may perform the role played by cyclic AMP in mammalian tissues. From the standpoint of effectiveness of kinase activation, this hypothesis is not supported by the experimental findings reported in this paper.

Materials and Methods

Materials

[γ-^{32}P] ATP was prepared essentially by published methods [10,11]. The incorporation of ^{32}P into ATP ranged from 80 to 90% and the specific activity varied from $3.1 \cdot 10^{11}$ to $4.8 \cdot 10^{11}$ dpm per mmole.

The following protein substrates for the kinase assay were purchased as lyophilized powders from Sigma: histone Type II-A (histone mixture); histone Type III (lysine rich); histone Type IV (arginine rich); α-casein; bovine serum albumin, fraction V (approx. 98% albumin); and protamine sulfate, Grade I from salmon. All histones were derived from calf thymus. A cyclic AMP-dependent protein kinase inhibitor purified from bovine heart was obtained from Sigma.

Enzyme preparation

Manduca sexta nerve cords were dissected and pooled on dry ice as described previously [12]. Homogenization of tissues (25—40 μl buffer per nerve cord) was performed in all-glass tissue grinders in 50 mM Tris—HCl, pH 7.0, containing 1 mM dithiothreitol and 1 mM $MgSO_4$. A 105 000 $\times$ *g* larval central nervous system supernatant was employed as the enzyme source in most experiments and was prepared by sedimenting the homogenate at 4°C for 2 h in a screw-capped polycarbonate centrifuge tube containing nitrogen gas. Protein was determined by the method of Lowry et al. [13].

Protein kinase assay

Protein kinase (ATP: protein phosphotransferase, EC 2.7.1.37) activity was assayed by measuring the enzyme catalyzed transfer of ^{32}P-labeled phosphate from [γ-^{32}P] ATP to phosphate acceptor protein [14]. Histone was used

as a substrate because the results were more reproducible and precise than with protamine as a substrate. The reaction conditions are given in Table I. A level of cyclic AMP approximately 100 fold greater than required for activation and short incubation times were used to minimize phosphodiesterase effects particularly in crude preparations.

After incubation the reaction vessels were plunged into an ice bath, and to each was added 2.0 ml of an ice-cold 20% (w/v) trichloroacetic acid solution containing 1 M H_3PO_4, followed by the addition of 0.01 ml of 0.1 M ATP. The resulting suspension was allowed to stand at 0—4°C for at least 10, but not more than 60 min prior to filtering on a Millipore filter (HAWP 02500, 0.45 μm). It was crucial to filter the precipitated phosphoprotein as rapidly as possible and not allow the filter to become dry during the initial stages of washing. The filter was thoroughly washed with 20 ml of cold 10% trichloroacetic acid, dried, then heated in a scintillation vial at 50°C for about 30 min with 1.0 ml of methylcellosolve. When the filter had completely dissolved, 10 ml of Aquasol was added to the vial and the solution was vigorously agitated for 2 h before counting in a Packard 2425 liquid scintillation spectrometer.

One unit of kinase activity is defined as that amount of enzyme which transfers one pmole (10^{-12} mole) of phosphate from ^{32}P-labeled ATP to protein acceptor in 5 min at 35°C under the assay conditions described.

Preparation of protein kinase inhibitor

Several tissues have been shown to contain a protein which inhibits the activity of cyclic AMP-dependent protein kinase [15—18]. The highest levels of this inhibitor have been found in mammalian brain tissue [15,16] and it was of interest to establish if such a factor were present in the insect central nervous system.

Essentially the procedure of Appleman et al. [19] and Gilman [20] was used to prepare a crude inhibitor fraction from *M. sexta* larval nerve cords. In brief, 52 larval nerve cords were homogenized in 2.0 ml of 10 mM Tris—HCl (pH 7.0)—2 mM EDTA, boiled at 100°C for 10 min, and centrifuged. The precipitate that formed after the addition of trichloroacetic acid (final concentration, 5%) was collected by centrifugation, suspended in buffer, dialyzed, and the dialyzed solution adjusted to pH 7.5 with 1 M NaOH, and lyophilized. The lyophilized powder was dissolved in 0.30 ml of neutral 10 mM Tris buffer to yield a solution containing 7.3 mg of the crude inhibitor fraction per ml.

A crude mammalian inhibitor fraction was prepared from 940 g of rabbit skeletal muscle through the dialysis step of the procedure described by Walsh et al. [15]. The insect nerve cord inhibitor fraction, as well as the mammalian preparations, were devoid of protein kinases and phosphoprotein phosphatase activities.

Results

Miscellaneous observations

Only a small fraction (less than 0.5%) of the total radioactivity present in each reaction mixture was incorporated into histone. The ^{32}P-labeled protein product was stable to boiling in neutral buffer, but was completely released by

heating for 10 min in 1 M NaOH indicating that the phosphate was esterified to protein serine and threonine residues [21,22]. *M. sexta* kinase activity was destroyed after boiling for 5 min. Repetitively freezing and thawing freshly-prepared 105 000 × *g* nerve cord supernatants or storage of supernatants at −20°C for as long as 4 weeks had no effect upon the magnitude of histone kinase activity, its Mg^{2+} requirement, or its cyclic nucleotide stimulability.

At a concentration of 1 mM the following compounds had little or no effect on cyclic AMP-stimulated histone kinase activity: adenine, adenosine, 3′-AMP, phosphoserine, phosphothreonine, *p*-nitrophenyl-phosphate, glucose 1-phosphate, glucose 6-phosphate, potassium phosphate and theophylline. ADP served as a relatively potent inhibitor (75% at 1 mM) of the insect enzyme and 1 mM 5′-AMP resulted in a 50% inhibition. When GTP was present in equal amounts to [^{32}P]ATP (each 125 mM), the incorporation of ^{32}P into histone was reduced by a factor of two.

Histone kinase activity was observed over a wide range of pH values and in Tris—HCl was relatively invariant from pH 7 to 10. Appreciable activity remained even at pH 11. Activity in 63 mM sodium glycyl-glycine buffer at pH 7.5 was 31% greater than that in Tris—HCl of the same molarity at the same pH. At pH 7.0, activity in 63 mM Tris—HCl was about 17% lower than in 31 mM Tris—HCl.

Phosphorylation of endogenous proteins

The endogenous kinase activity of high speed supernatants varied from 5 to 30% of the total activity observed with 250 μg of histone in the presence of 10^{-5} M cyclic AMP. The extent of phosphorylation of endogenous substrates in eleven different experiments ranged from 350 to 950 units of activity per mg of endogenous protein, and the influence of cyclic AMP or cyclic GMP on the phosphorylation of supernatant proteins was not consistent as observed in other systems [23,24].

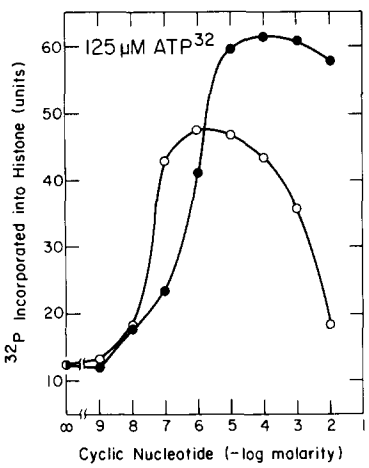
There occurred a 40—50% loss in endogenous kinase activity after rapidly freezing and thawing fresly-prepared supernatants a single time.

Sulfhydryl sensitivity

When 1 mM dithiothreitol was included in both homogenizing and assay buffers, kinase activity was enhanced four-fold as found in trout testis [25]. Cysteine was not nearly as effective and mercaptoethanol had no effect. *p*-Hydroxymercuribenzoate inhibited cyclic AMP-dependent histone kinase activity in 105 000 × *g* supernatants by 10% at 10^{-5} M, 78% at 10^{-4} M, and 95% at 1 mM. At a given concentration of *p*-hydroxymercuribenzoate, cyclic AMP-dependent was inhibited more than cyclic AMP-independent activity. It is thus evident that the soluble kinase(s) of the *M. sexta* nerve cord require a reduced sulfhydryl group for maximum activity in contrast to most cyclic AMP-dependent protein kinases [1]. Donnelly et al. [26] reported that a cyclic GMP-dependent protein kinase from bovine heart were inactivated by *p*-hydroxymercuribenzoate.

Effectiveness of cyclic AMP and cyclic GMP in activating soluble protein kinase

The effects of different concentrations of cyclic AMP and cyclic GMP on



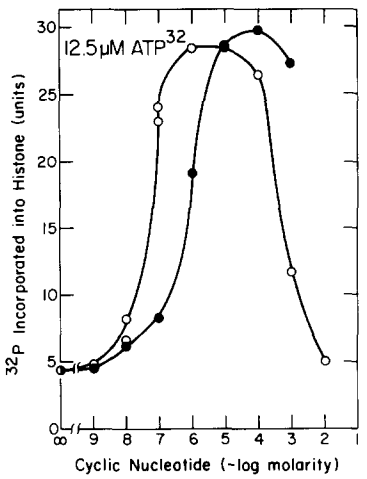


Fig. 1. Cyclic nucleotide dependence of histone kinase activity. Each reaction mixture contained 19.6 μg of 105 000 × *g* larval central nervous system supernatant, 250 μg histone, 125 μm ATP, and cyclic AMP or cyclic GMP as indicated. Incubation conditions are described in Table I and the correction for endogenous phosphorylation was 14.5 units. ○———○, cyclic AMP; ●———●, cyclic GMP.

Fig. 2. Cyclic nucleotide dependence of histone kinase activity. Experimental conditions were the same as described in the legend to Fig. 1 except that 12.5 μM of ATP was added, and the correction for endogenous phosphorylation was 5.1 units. ○———○, cyclic AMP; ●———●, cyclic GMP.

the phosphorylation of histone in the presence of either 12.5 μM or 125 μM [^{32}P] ATP were determined (Figs 1 and 2). At both concentrations of ATP the concentration of cyclic AMP required to produce half-maximal stimulation was between 10- and 100-fold lower than that of cyclic GMP. At a sufficiently high level of cyclic AMP (approx. 1 μM), the rate of histone phosphorylation was increased by about four-fold in the presence of 125 μM ATP and by about six-fold in the presence of 12.5 μM ATP. When the concentration of cyclic AMP was increased past 10^{-5} M, the degree of kinase stimulation rapidly declined, possibly due to competition of cyclic AMP with ATP for enzyme catalytic sites. At concentrations of cyclic nucleotide greater than 10^{-4} M in the presence of 125 μM ATP (Fig. 1), cyclic GMP became much more effective than cyclic AMP in activating soluble protein kinase. However, at an ATP level of 12.5 μM and cyclic nucleotide levels greater than 10^{-5} M, cyclic GMP was not more effective (Fig. 2). This lower ATP level may be more reflective of the in vivo situation.

Activation profiles similar to those shown have been reported for numerous impure and partially purified cyclic AMP- and cyclic GMP-dependent protein kinases [2,4,8,27—29].

Effect of varying enzyme, histone, or ATP concentrations

The phosphorylation of histone at an ATP concentration of 125 μM, with or without cyclic AMP present, was directly proportional to the amount of 105 000 × *g* supernatant protein assayed up to a concentration of 20 μg per reaction mixture (0.1 mg/ml) and departed only slightly from linearity up to

60 g of protein. The phosphorylation of endogenous proteins was linear with increasing amounts of added supernatant up to about 20 μg of protein.

Double-reciprocal plots of the phosphorylation of histone (50—750 μg per reaction mixture) by soluble nerve cord kinase at an ATP concentration of 125 μM were linear and indicated a K_m for histone of 80 μg per reaction mixture (0.4 mg/ml) both in the presence and absence of 10^{-5} M cyclic AMP as reported for mammalian systems [3,30]. In the presence of cyclic AMP, the V of the insect kinase was increased by about three-fold.

Kinase activity as a function of ATP concentration

The dependence of the reaction velocity on the concentration of ATP was examined (Fig. 3). In the presence of 10^{-5} M cyclic AMP and 500 μM ATP there occurred an apparent stimulation of kinase activity. The time course of phosphorylation observed under these conditions also revealed a marked deviation from linearity after 4 min of incubation. These effects at high ATP concentrations may be due to a high K_m protein kinase which is not saturated at the comparatively low ATP level (125 μM) employed in routine assays. Alternatively, a low K_m kinase in the presence of cyclic AMP and high levels of ATP may undergo a time-dependent activation.

After correction for endogenous phosphorylation, double-reciprocal plots of the data of Fig. 3 were nonlinear and showed progressive downward curvature as the concentration of ATP increased. From the activities at ATP concentrations in the range 12.5—250 μM, K_m values in the presence and absence of 10^{-5} M cyclic AMP were approximately 10 μM and 50 μM, respectively. In addition to lowering the apparent K_m by a factor of five, cyclic AMP resulted in an approximate doubling of the V. These results are similar to those found in brain [3,30] but not in non-neural tissues [30—32].

Kinase activities in the larval and adult central nervous system

In Table I are collected representative data from different experiments on supernatants prepared from *M. sexta* nerve cords. Larval central nervous system 10 000 × *g* supernatants exhibited somewhat greater specific activities than

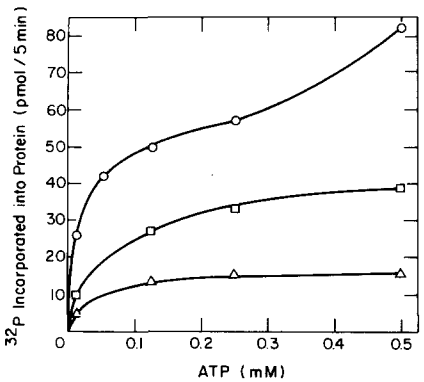


Fig. 3. Kinase activity as a function of ATP concentration. Each reaction mixture contained 20.6 μg of protein from a 105 000 × *g* supernatant. Assay conditions are described in Table I. ○———○, 10^{-5} M cyclic AMP; □———□, no cyclic AMP; △———△, endogenous phosphorylation.

TABLE I

PROTEIN KINASE ACTIVITIES IN *M. SEXTA* LARVAL AND ADULT CENTRAL NERVOUS SYSTEM SUPERNATANTS

Nerve cord homogenates were prepared as described in Materials and Methods and centrifuged at either 10000 × *g* for 10 min or 105000 × *g* for 2 h before assay of the supernatant. The reaction mixture contained 50 mM Tris—HCl, 5 mM $MgSO_4$, 5 mM dithiothreitol, 250 μg of histone II-A, 125 μM [^{32}P]-ATP, 10^{-5} M cyclic AMP as indicated, approximately 40 μg of enzyme protein in a total volume of 0.2 ml, pH 7.0. Each reaction was initiated by the addition of enzyme protein. Incubation was in a shaking water bath at 35°C for 5 min. Activities were not corrected for endogenous phosphorylation.

Experiment	No. of central nervous system	Enzyme source	10^{-5} M cyclic AMP	Specific activity (units/mg protein)	Activity per nerve cord (units/central nervous system)	Ratio +/−
Larval central nervous system						
1a	30	10000 × *g* supernatant	+	4411	601	—
1b		105000 × *g* supernatant	+	3933	313	
			−	1803	143	2.19
2	10	10000 × *g* supernatant	+	5495	559	
			−	2364	240	2.33
3	40	105000 × *g* supernatant	+	2859	243	
			−	1104	94	2.59
4	40	105000 × *g* supernatant	+	3454	259	
			−	1685	126	2.05
5	45	105000 × *g* supernatant	+	4072	305	
			−	1571	118	2.58
6	12	105000 × *g* supernatant	+	3556	257	
			−	1711	124	2.07
Adult central nervous system						
7	8	105000 × *g* supernatant	+	8625	707	
			−	1587	130	5.43

105 000 × *g* supernatants. Among those experiments summarized in Table I, the mean specific kinase activity of larval central nervous system high-speed supernatants was 3574 and 1575 units/mg protein, respectively, in the presence and absence of cyclic AMP. In comparison, the adult central nervous system supernatant specific kinase activity with cyclic AMP present was considerably higher (8625 units/mg), but cyclic AMP-independent activity in this tissue (1587 units/mg) was approximately the same as that of the larval central nervous system. The ratio of activities (+cyclic AMP/—cyclic AMP) in the adult central nervous system supernatant was more than twice that of the larval nerve cord.

If freshly-prepared larval central nervous system 10 000 × *g* supernatants were allowed to "age" at 25°C for various periods of time before assay, histone kinase activity increased almost linearly with time until 60 min had elapsed, at which time activity in the presence of cyclic AMP had almost doubled relative to that measured immediately after preparation. Increases occurred in both cyclic AMP-dependent and cyclic AMP-independent activities, but the magnitude of kinase stimulation by cyclic AMP at zero time (× 2.6) was much greater than that after 60 min of aging (× 1.5). This aging phenomenon did not occur in 105 000 × *g* supernatants. This gradual increase in activity in low speed supernatants may be due to a time-dependent activation of kinase in membrane fractions or a slow release of activity from particulate material.

Histone kinase activity could be solubilized from central nervous system particulate fractions by detergent treatment. 30 larval nerve cords were homogenized and centrifuged at 105 000 × *g* for 2 h. The 105 000 × *g* pellet was homogenized for 1 min at 4° C in Tris buffer containing 1% (w/v) Triton X-100 and centrifuged again for 2 h. The resulting supernatant contained an additional cyclic AMP-dependent protein kinase activity equal to 70% of that found in the initial 105 000 × *g* extract.

This particulate-associated but detergent extractable kinase exhibited a cyclic nucleotide activation profile (Fig. 4) quite distinct from that found for soluble kinase (Fig. 1). The ascending segment of the cyclic AMP activation curves (10^{-8}—10^{-6} M cyclic AMP) were similar as was the concentration of cyclic AMP (approx. 10^{-6} M) which promoted greatest activity in both samples. However, in the kinase extracted from the 105 000 × *g* pellet, (a) activation was clearly evident at the very low cyclic AMP concentrations less than 10^{-6} M, (b) activity declined rapidly at cyclic AMP concentrations greater than 10^{-6} M and (c) at 10^{-6} M, cyclic GMP was much less effective than cyclic AMP in stimulating activity. These features distinguish membrane-associated kinase from soluble kinase.

Several experiments established that Triton X-100 at concentrations up to 5% (w/v) did not affect the magnitude of activity or cyclic nucleotide stimulability of kinase in 105 000 × *g* supernatants. However, this detergent was capable of unmasking considerable additional kinase activity from particulate material present in 1000 × *g* and 10 000 × *g* supernatants.

Effects of univalent salts

Several investigators [32,33] have shown that univalent salts inhibit protein kinase activity by decreasing its affinity for its protein substrate [34]. These inhibitory effects were also noted for soluble protein kinase from the insect nerve cord (Fig. 5).

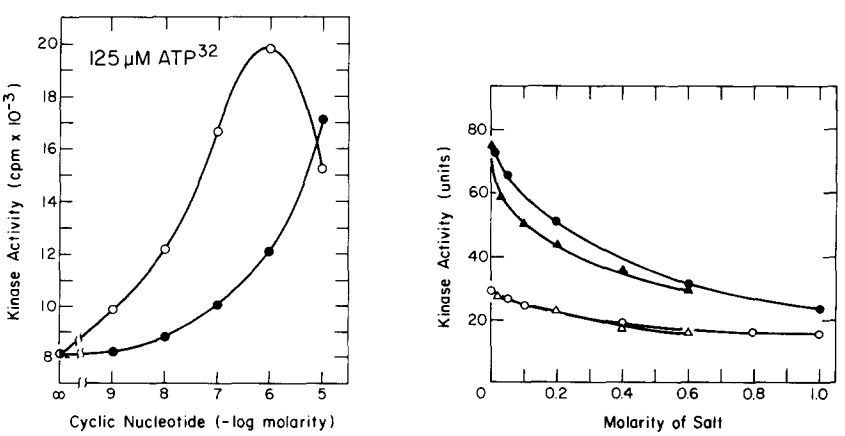


Fig. 4. Cyclic nucleotide dependence of kinase activity extracted from the 105 000 × *g* pellet with Triton X-100. Incubation conditions are described in Table I. ○———○, cyclic AMP; ●———●, cyclic GMP.

Fig. 5. Inhibition of kinase activity by NaCl and KCl. Each reaction mixture contained 19.6 μg of protein from a 105 000 × *g* supernatant. The sum of endogenous plus exogenous (histone) kinase activity is indicated. Endogenous activity in the absence of salt was 15.2 units in the presence of cyclic AMP. ●———●, NaCl + cyclic AMP; ○———○, NaCl, no cyclic AMP; ▲———▲, KCl + cyclic AMP; △———△, KCl, no cyclic AMP.

Both NaCl and KCl inhibited cyclic AMP-dependent activity to a much greater extent than cyclic AMP-independent activity. With 10^{-5} M cyclic AMP present, KCl was more inhibitory than NaCl at any concentration, but these two salts were indistinguishable in their influence on kinase activity in the absence of cyclic AMP. LiCl was more inhibitory than KCl. With increasing salt concentration the ratio of activities in the presence and absence of cyclic AMP progressively decreased, which may indicate that high ionic strength conditions inhibit cyclic AMP binding to a regulatory subunit of protein kinase.

Effects of bivalent cations and EDTA

Both endogenous and exogenous kinase activities were strongly activated by Mg^{2+} and completely abolished with EDTA, indicating that nerve cord kinase is Mg^{2+}-dependent. Mn^{2+}, Cu^{2+}, and Fe^{2+} had little effect on the insect enzyme. Ca^{2+} and Zn^{2+} were inhibitory. At 5 mM, Co^{2+} was about half as effective as Mg^{2+} in stimulating activity. Activation of kinase by Mg^{2+} in the presence or absence of cyclic AMP was appreciable at 0.5 mM, optimal at 5 mM, and declined at higher concentrations. The enzyme activity vs. Mg^{2+} concentration relationship determined for *M. sexta* central nervous system kinase was similar to those reported for kinases from calf thymus nuclei [35], bovine muscle [30] and bovine brain [32].

Abilities of various proteins to serve as kinase substrates

A number of proteins were examined for their ability to serve as substrates for nerve cord kinase (Table II), and of these, protamine was the most

TABLE II

ABILITIES OF VARIOUS PROTEINS TO SERVE AS SUBSTRATES FOR SOLUBLE PROTEIN KINASE FROM THE *M. SEXTA* LARVAL CENTRAL NERVOUS SYSTEM

Incubations were conducted as described in Table I and other methods, except for the variation of kind and amount of substrate. Each reaction mixture contained 36.4 μg of a 105 000 × *g* supernatant. Activities in the presence of exogenous substrate have not been corrected for endogenous phosphorylation.

Exogenous substrate	Amount (μg)	Kinase activity (units)		Ratio +/-
		- cyclic AMP	+ cyclic AMP	
None		14.5	15.3	1.05
Histone II-A*	250	54.0	124.8	2.31
	50	30.1	65.5	2.18
Histone III*	250	45.4	88.4	1.95
	50	39.7	47.7	1.20
Histone IV*	250	50.8	103.0	2.03
	50	30.1	53.2	1.77
Protamine	250	300.4	477.3	1.59
	50	101.5	184.1	1.81
Bovine serum albumin (fraction V**)	250	31.5	32.4	1.03
	50	15.9	19.8	1.17
α-casein	250	44.0	44.2	1.00
	50	32.1	26.8	0.83

* For brevity of description, Sigma "Type" designations are indicated with roman numerals. II = histone mixture, III = lysine-rich, IV = arginine-rich histone.

** 98% albumin.

readily phosphorylated both in the presence and absence of cyclic AMP. The various types of histone tested were roughly comparable as substrates for soluble kinase. In contrast to other results with non-mammalian kinase preparations [3], casein is a less effective substrate than histone for the insect kinase (Table II).

A great variation in the effect of cyclic AMP on the phosphorylation of the different proteins was observed. Stimulation of kinase activity by 10^{-5} M cyclic AMP was greatest for histone II (mixed histones), and decreased in the order histone II, histone IV, histone III, protamine, serum albumin and casein. If endogenous kinase activity were subtracted from the total (endogenous + exogenous) activity in the presence of added substrate, even greater values than those shown in Table II for the degree of stimulation by cyclic AMP would be calculated. Cyclic AMP had little effect on the phosphorylation of albumin and appeared to slightly inhibit the phosphorylation of casein as reported in other systems [21,24,36,37].

Protein kinase inhibitor

As is indicated in Fig. 6, some component of the insect protein kinase inhibitor fraction (see Materials and Methods) seriously interfered with soluble cyclic AMP-dependent protein kinase activity of the larval central nervous system. The degree of inhibition rose sharply with increasing amounts of inhibitor and then apparently reached a plateau level (about 10% of the activity measured with no inhibitor present) at higher concentrations. When present at 250 μg per ml in the reaction mixture, the mammalian kinase inhibitor fractions derived from bovine heart and rabbit muscle were totally without effect on the insect kinase cyclic AMP-dependent activity.

Assuming a 100% recovery of inhibitor activity in the lyophilized inhibitor fraction (i.e. all of the activity present in the nerve cord starting material), it was calculated that the amount of this fraction required to produce 50% inhibition of high speed supernatant cyclic AMP-dependent protein kinase activity was only about twice that amount already contributed to the reaction mixture by the quantity of supernatant assayed. Thus, kinase activity in the

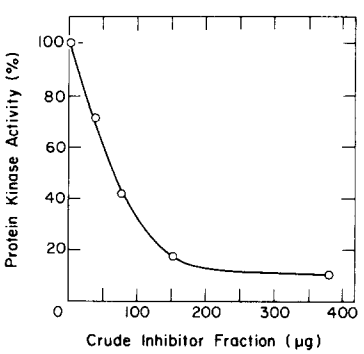


Fig. 6. Effect of the insect crude inhibitor fraction on soluble cyclic AMP-dependent kinase activity. Each reaction mixture contained 46 μg protein from a 105 000 × *g* supernatant, 250 μg of histone and 27 μM [α-^{32}P]ATP. Activity is expressed as per cent of that observed when no inhibitor is present.

crude soluble fractions used for the present studies of the *M. sexta* central nervous system was highly suppressed by endogenous inhibitor.

Polyacrylamide disc gel electrophoresis

Preliminary experiments indicate the presence of multiple protein kinases in soluble extracts from the larval central nervous system. A 35 000 × *g* supernatant was separated on a polyacrylamide disc gel essentially by the method of Davis [38] and after electrophoresis, the separating gel was frozen and cut into small slices. Each slice was homogenized in buffer, centrifuged and assayed for histone kinase activity. Activity was distributed from the separating gel—stacking gel interface (R_f = 0) to a position in the gel corresponding to R_f = 0.6, with two major bands present at R_f = 0.33—0.37 and two less active bands at R_f = 0.50—0.53. Two of the bands (R_f = 0.33, 0.50) were weakly stimulated by cyclic AMP, but the other two were not. These observations are compatible with the hypothesis that those slices stimulated by cyclic AMP represent kinase holoenzymes (catalytic plus regulatory subunits), whereas those not affected by cyclic AMP represent either catalytic subunits of cyclic AMP-dependent enzymes or non-cyclic nucleotide-sensitive protein kinases.

Discussion

Protein kinases of the *M. sexta* central nervous system have been shown to be more effectively activated by low concentrations of cyclic AMP than by cyclic GMP. This contrasts with the behavior of other Lepidopteran kinases reported by Kuo et al. [4]. However, the existence in the central nervous system of cyclic AMP-specific, cyclic GMP-specific, and possibly cyclic nucleotide-independent protein kinases remains to be established. Since considerable kinase activity was measured in vitro in the absence of added cyclic nucleotide, one might infer the presence of kinases not regulated by cyclic AMP or cyclic GMP. Yet these activities could also be attributed to catalytic kinase subunits dissociated from the holoenzyme either in vivo or during the process of preparation.

Although the experiments described were largely constrained to an examination of several enzymological properties of soluble protein kinase, it was determined that a substantial amount of activity is present in particulate fractions of the *M. sexta* central nervous system. Latent activity could be unmasked from these fractions by incubation with Triton X-100, which presumably lyses organelles and also solubilizes membrane-associated kinase. Triton-extracted kinase activity exhibited an activation profile in the presence of cyclic AMP and cyclic GMP which distinguished it from soluble kinase. Of emphasis is the fact that for these studies nervous tissues were stored frozen and then homogenized in hypotonic buffer, and it is likely that vesicular structures were osmotically and mechanically ruptured. Assay of supernatants and pellets prepared in this fashion would result in a conservative estimate of the activity associated with particulate material in vivo and overestimation of cytosolic activity. Further subcellular fractionation studies of *M. sexta* nervous tissues should be conducted to establish if, as is the case with rat brain, the majority of protein kinase activity is concentrated in synaptosomes, synaptic membrane fragments and synaptic vesicles [23].

In general, the enzymological properties of the insect central nervous system kinase are similar to those reported for other kinases with respect to activation by cyclic AMP and cyclic GMP, Mg^{2+} requirement, inhibition by ADP, K_m for exogenous protein substrate, influence of univalent salts, inhibition by a heat-stable factor (protein kinase inhibitor) and lack of stimulation by cyclic AMP of casein phosphorylation. The *M. sexta* kinase appeared to differ from mammalian kinases in that GTP was a very effective inhibitor of ATP:protein phosphotransferase activity. This finding suggests that the nucleoside triphosphate substrate specificity of nerve cord kinase should be investigated to establish the existence and properties in this tissue of ATP-specific kinases, GTP-specific kinases, and/or ATP and GTP-nonspecific kinases regulated by either cyclic AMP or cyclic GMP.

The cyclic AMP and cyclic GMP content of larval *M. sexta* nerve cords has been determined by competitive protein binding radiometric techniques (unpublished). Assuming that water constitutes 80% by weight of the insect central nervous system and that the cyclic nucleotides are uniformly distributed throughout the aqueous phase, the basal intracellular levels of cyclic AMP and cyclic GMP were estimated to be 0.7 μM and 7 μM, respectively. Local concentrations of these cyclic nucleotides may, however, be much higher or lower than these values. In any case, levels of cyclic AMP and cyclic GMP of this order of magnitude would appreciably activate soluble cyclic nucleotide-stimulable kinase in the central nervous system of *M. sexta*.

Acknowledgments

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