Properties of Adenosine 3',5'-Monophosphate-dependent Protein Kinases Isolated from Bovine Epididymal Spermatozoa*

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SUMMARY

Three protein kinases were separated from bovine epididymal spermatozoa by using DEAE-Sephadex ion exchange chromatography and Sephadex G-100 gel filtration. The molecular weights of these three kinases were estimated to be 120,000, 78,000 and 56,000 by the use of Sephadex G-100 gel filtration. The activity of all three kinases was greatly enhanced by cyclic adenosine 3',5'-monophosphate (cyclic AMP). In the presence of 10⁻⁵ M cyclic AMP, a catalytic subunit of molecular weight 30,000 to 35,000 was separated from each of the three protein kinases. Under these same conditions, cyclic AMP-binding proteins were located in fractions corresponding to molecular weights of 78,000, 35,000 to 40,000, and 17,000 to 18,000; the latter may result from proteolysis for it was not observed in fresh preparations. The cyclic AMP-binding protein of molecular weight 78,000 combined with the isolated catalytic subunits of all three protein kinases.

The apparent Kₐ for casein (approximately 3 mg per ml) appeared to be neither different for the three kinases nor affected by the addition of cyclic AMP. The apparent Kₐ for ATP varied from 3.5 to 8.7 μM, but it appeared to be the same for all three protein kinases and also appeared to be unaffected by cyclic AMP. The isolated catalytic subunit of the kinase of molecular weight 120,000 exhibited a Kₐ for ATP of 5.5 μM, which agreed with the Kₐ for the intact kinase.

Maximum kinase activity was at about pH 6.5 with casein as substrate and at about pH 7.5 with histone as substrate for all three kinases. The isolated catalytic subunit of the 120,000 molecular weight kinase showed identical responses to pH.

Cyclic AMP activated all three kinases half-maximally in the 10⁻⁷ M range, whereas cyclic GMP activated the kinases half-maximally in the 10⁻⁸ M range. 8-Methylthio-cyclic AMP appeared to be more potent than cyclic AMP in activating the kinases.

The data suggest that the catalytic subunits of the three kinases are either similar or identical.

Motility can be induced and prolonged in the spermatozoa of several mammalian species by cyclic nucleotides (1-4). This stimulatory effect on motility is not surprising since the inotropic effects of catecholamines on cardiac muscle (5, 6), the β-adrenergic relaxing effects in most smooth muscle (7), the contact inhibition of motility in cultured fibroblasts (8), and the failure of adenylate cyclase-deficient bacterial mutants to develop functional flagella (9) seem to be mediated by the presence, and in the latter case the absence, of cyclic adenosine 3',5'-monophosphate.

The regulation of spermatozoan motility and metabolism by cyclic nucleotides must be considered as potentially important to understanding the processes of sperm activation, ovum penetration, and eventual fertilization. Yanagimachi (10, 11) has reported that capacitated hamster sperm acquire a more rapid motility that is still exhibited by sperm penetrating the ovum. (Capacitation is the process requiring time of sperm residence in the female reproductive tract prior to their acquiring the ability to penetrate the ovum.) A number of reports have also claimed that marked acceleration of sperm respiration occurs in capacitated rabbit sperm (12-14). Castañeda and Tyler (15) indicated that adenylate cyclase activity of sea urchin eggs increased upon fertilization.

We have found bovine epididymal sperm to contain cyclic AMP-dependent protein kinases and report in this paper the general characteristics of these enzymes.

EXPERIMENTAL PROCEDURES

Materials

Casein (Calbiochem) was dissolved by the methods of Riemann et al. (16). Histone was from Calbiochem; cyclic [³H]AMP from New England Nuclear; other cyclic nucleotides from Boeh-

* The abbreviations used are: cyclic AMP, cyclic adenosine 3',5'-monophosphate; 8-dimethyIamino-cyclic AMP, 8-(dimethylamino)cyclic adenosine 3',5'-monophosphate; 8-methylthio-cyclic AMP, 8-(methylthio)cyclic adenosine 3',5'-monophosphate; cyclic GMP, cyclic guanosine 3',5'-monophosphate.
ringer Mannheim or Calbiochem; cyclic AMP analogues (SQ 80001 and SQ 80002) supplied by Dr. S. M. Hess of the Squibb Medical Institute; DEAE-Sephadex and Sephadex G-100 from Pharmacia; [γ-32P]ATP was prepared by the procedure of Penefsky (17) by Dr. Rainer Zahlten of this laboratory; Millipore filters (0.3-μm pore size, 25-mm diameter) from Millipore, Inc.; glass fiber filters (type E) from Gelman Instrument Company; bovine epididymides from Oscar Mayer and Company, Madison, Wisconsin.

Methods

Assay of Cyclic AMP-binding Activity—Twenty-five microliters of cyclic [3H]AMP (22.7 pmoles), 15 μl of protein (0 to 100 μg) solution, and 0.21 ml of 200 mM EDTA, 50 mM acetate at pH 6 were incubated at 0°C for about 1 hour. These reaction mixtures were then washed onto Millipore filters with 10 ml of 200 mM EDTA, 50 mM acetate at pH 6. The high concentration of EDTA does not interfere with cyclic AMP binding but completely inhibits the cyclic nucleotide phosphodiesterase. The collected filters were dried and counted in a liquid scintillation counter using 10 ml of a solution containing 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl POPPO) and 4 g of 2,5-diphenyloxazole (PPO) per liter of toluene.

Assay of Protein Kinase Activity—Protein kinase activity in column effluent fractions was estimated by using histone as the substrate. Histone itself activated the protein kinases and was replaced by casein when kinetic characteristics of the kinases were determined. Our standard assay consisted of 77 μmol [γ-32P]ATP, 7.5 mM MgCl₂, enzyme, 0.2 mg of histone, and 33 mM Tris-Cl, pH 7.5, in a total volume of 0.15 ml. The reaction at 30°C was started by addition of ATP and allowed to proceed for 5 or 10 min; reactions were linear for at least 15 min. Reactions were terminated by addition of 0.5 ml of 10% trichloroacetic acid. Bovine serum albumin solution (12.7 ml) was added to each tube. After tubes were chilled on ice for about 15 min, the precipitated protein was pelleted by centrifugation and the supernatant fluid removed. The pellets were dissolved in 0.1 ml of 1 N NaOH then were precipitated again with 1.0 ml of 10% trichloroacetic acid. After 15 min on ice, the samples were collected and washed with 20 ml of 10% trichloroacetic acid onto glass fiber filters. These filters were dried and counted in a liquid scintillation counter using 10 ml of a solution containing 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl POPPO) and 4 g of 2,5-diphenyloxazole (PPO) per liter of toluene.

Protein Determination—Both biuret and absorption at 280 nm were used to estimate protein concentrations (18). Prior to estimation of protein in a sample, the sample was dialyzed against distilled water for 12 hours at 2 to 4°C to eliminate interference from Tris or dithiothreitol.

Results

Partial Purification and Separation of Protein Kinases

Recovery of Soluble Protein—Bovine epididymal sperm (2.1 X 10⁶; approximately 15.4 g wet weight) were collected and washed with a modified Krebs-Ringer buffer (120 mM NaCl, 5 mM KCl, 10 mM KH₂PO₄, 5 mM MgSO₄, and 10 mM Tris-Cl at pH 7) as described previously (1). The washed sperm pellet was resuspended in 50 mM Tris-Cl, 1 mM dithiothreitol at pH 7 and sonicated at three intervals for 0.5 to 1.0 min on a Branson sonifier (model LS-75) at full amperage at 0°C. This treatment causes tail disintegration on all sperm but does not cause release of DNA from sperm heads. Centrifugation at 15,000 X g for 30 min to remove most of the particulate material followed by centrifugation of this supernatant fluid at 85,000 X g for 1 hour resulted in a clear supernatant solution of the crude soluble protein kinases. This fluid was dialyzed against 50 mM Tris-Cl, 1 mM dithiothreitol at pH 7 for approximately 12 hours at 2 to 4°C.

DEAE-Sephadex Elution—A DEAE-Sephadex A-50 column (2.5 X 38 cm) was equilibrated with 50 mM Tris-Cl, 1 mM dithiothreitol at pH 7. The dialyzed soluble sperm extract (127 ml) containing 331 mg of protein was applied to the column and, after washing the column with about 10 ml of the same buffer, a linear gradient was started. To make the gradient, 250 ml of 50 mM Tris-Cl, 1 mM dithiothreitol at pH 7 in the mixing chamber and 250 ml of the same buffer containing 0.5 M KCl in the opposite chamber were used. Both cyclic [3H]AMP binding and protein kinase activity were measured on the 4.5-ml effluent fractions. Two peaks of activity were consistently eluted (Fig. 1). The peaks were designated as kinases I and II and were pooled as indicated. Kinase I was precipitated by the addition of 0.34 g of (NH₄)₂SO₄ per ml, and 0.5 g of (NH₄)₂SO₄ per ml was used to precipitate kinase II. The proteins were stored at −10°C in (NH₄)₂SO₄ and were pelleted by centrifugation and diluted with 50 mM Tris-acetate, 1 mM dithiothreitol at pH 7 prior to gel filtration.

Gel Filtration I—Kinase I (54 mg of protein in 4.0 ml) or kinase II (66 mg of protein in 4.0 ml) was added to a Sephadex G-100 column (2.5 cm X 100 cm) equilibrated with the Tris-acetate, dithiothreitol buffer mentioned above. Kinase I eluted as two peaks; they were designated as IA and IB (Fig. 2). The cyclic AMP-binding activity co-eluted with the kinase activity. The elution pattern shown in Fig. 2 was obtained with several batches of sperm extract. However, in one experiment, Peak IB was only a minor component.

FIG. 1 (left). The elution of bovine epididymal sperm protein kinase and [3H]cyclic AMP-binding activities from a DEAE-Sephadex column (2.5 X 38 cm). Conditions were as described in the text. □—□, cyclic AMP-binding activity; —-, absorbance at 280 nm.

FIG. 2 (right). Gel filtration of kinases I or II prepared by DEAE-Sephadex ion exchange chromatography. Application of the enzyme to the Sephadex G-100 column, and assay methods for protein kinase and cyclic AMP binding are as described in the text. Δ—Δ, cyclic AMP binding; O—O, protein kinase activity. The center scale applies to both charts.
on 57, polyacrylamide gel, yielded a single band of protein that IA was eluted from Sephadex G-100 in the presence of 1 μM cyclic 

purified C proteins, when subjected to electrophoretic separation spermatozoa is represented by the three protein kinases. The 

35,000 to 40,000 (R-2) and at 17,000 to 18,000 (R-3) when kinase 

ment described. Nearly 10% of the soluble protein of the bovine 

as C-IA, had an estimated molecular weight of 30,000 to 35,000, 

about 120,000. The catalytic protein of kinase IA, designated 

with the same concentration of ammonium sulfate but in the 

SO4 per ml at 0". The other protein factors were precipitated 

cipitated with ammonium sulfate. The large molecular weight 

cyclic AMP-binding protein of kinase IA and the catalytic unit 

weight were made using the gel filtration data described in the 

Gel Filtration II—A sephadex G-100 column (2.5 cm × 100 

cm) was equilibrated with the 50 mM Tris-acetate, 1 mM dihydrothreitol buffer described previously, except that 1 μM cyclic AMP 

was included in the buffer. Kinases IA (12 mg of protein, 2 ml), IB (3.3 mg of protein, 2 ml) or II (6.7 mg/2 ml) were applied to the Sephadex G-100 column (Fig. 3). In the presence of cyclic AMP, the catalytic activity now filtered as a small molecular weight protein. With all three kinase preparations, catalytic activity eluted in the same fractions. Cyclic AMP-binding activity migrated as three peaks when kinase IA was filtered on Sephadex G-100, as two peaks when kinase II was filtered, and without detection when kinase IB was filtered.

To stabilize the proteins for storage at −10°, they were precipitated with ammonium sulfate. The large molecular weight cyclic AMP-binding protein of kinase IA and the catalytic unit of kinase IA were precipitated by the addition of 0.5 g of (NH₄)₂ SO₄ per ml at 0°. The other protein factors were precipitated with the same concentration of ammonium sulfate but in the presence of 0.25 mg of bovine serum albumin per ml.

Table I demonstrates the percentage of recovery and the specific activities of the various fractions obtained in the experiment described. Nearly 10% of the soluble protein of the bovine spermatozoa is represented by the three protein kinases. The purified C proteins, when subjected to electrophoretic separation on 5% polyacrylamide gel, yielded a single band of protein that possessed kinase activity. With heavily loaded gels, a faint second band was discernible. The latter protein bound cyclic AMP and was presumed to be R protein.

Properties of Sperm Protein Kinases

Molecular Weight Estimates—Estimates of enzyme molecular weight were made using the gel filtration data described in the purification sections. The Sephadex G-100 column was calibrated with four standard proteins (Fig. 4). Kinase IA eluted near the void volume with an extrapolated molecular weight of about 120,000. The catalytic protein of kinase IA, designated as C-IA, had an estimated molecular weight of 30,000 to 35,000, while the large regulatory protein, designated as R-1, had an estimated molecular weight of about 78,000. Cyclic AMP-binding protein also eluted at apparent molecular weights of 35,000 to 40,000 (R-2) and at 17,000 to 18,000 (R-3) when kinase IA was eluted from Sephadex G-100 in the presence of 1 μM cyclic AMP. On sodium dodecyl sulfate disc gel electrophoresis R-1 always migrated as a single band corresponding to a molecular weight of about 40,000. R-2 also formed a single band of the same mobility. Preparations that were not stored before gel filtration did not yield an R-3 component. It seems likely that native R-1 is a dimer, that R-2 is the protomer, and that R-3 is derived from the protomer by proteolysis during storage of the preparations.

The molecular weight of kinase IB was about 56,000. The catalytic unit of kinase IB had an estimated molecular weight of 30,000 to 35,000, but no cyclic AMP-binding activity was located after Sephadex G-100 filtration.

The estimated molecular weight of kinase II was about 78,000. Elution of kinase II on a Sephadex G-100 column in the presence of cyclic AMP yielded a catalytic unit at 30,000 to 35,000 and cyclic AMP-binding proteins at 35,000 to 40,000 (R-2) and at
The value in parentheses represents the apparent $K_v$, in the presence of $6.7 \times 10^{-5} M$ cyclic AMP (Fig. 5). The plots were linear to a concentration of 0.25 mg casein per ml. The Michaelis constants for protein kinases are profoundly affected by salt concentration (16). The determinations reported here were terminated, and the $32P$ incorporated into casein was determined.

Two and one-half micrograms of C-IA, 1 $\mu$g of C-IB, and 0.5 $\mu$g of C-II were added in the presence or absence of 50 $\mu$g of R-1. When added, cyclic AMP was present in a final concentration of $6.7 \times 10^{-5} M$. These solutions were allowed to incubate for 30 min on ice. The reactions were then started by the addition of the standard reaction mixture described in the text using casein as substrate. After incubation for 10 min at 30° the reactions were terminated, and the $32P$ incorporated into casein was determined.

### Table II

<table>
<thead>
<tr>
<th>Catalytic fraction</th>
<th>Addition of R-1</th>
<th>$32P$ incorporated</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cyclic AMP</td>
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<tr>
<td>C-IA</td>
<td>$-$</td>
<td>75.2</td>
</tr>
<tr>
<td>C-IB</td>
<td>$+$</td>
<td>10.7</td>
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<tr>
<td>C-II</td>
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<td>130.3</td>
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78,000 (R-1) molecular weights (Fig. 3). The R-2 component appears not to combine with the catalytic unit once it has been dissociated, for the C unit from kinase II is not stimulated by cyclic AMP (Table II).

pH—All three kinases exhibited optimum kinase activity at about pH 6.5 with casein as substrate. The optimum was shifted to pH 7.5 with histone as substrate. The isolated catalytic subunit of kinase I (C-IA) also exhibited maximal activity at pH 6.5 with casein and at pH 7.5 with histone as substrate.

Protein Substrate—It was noted early in this work that with histone as substrate, cyclic AMP activated the protein kinase only 2- to 3-fold. The basal kinase activity in the absence of cyclic AMP, however, was considerably greater than that with casein as substrate. This suggests that histone induces a dissociation of receptor protein from catalytic protein. Recent evidence suggests that histones (19) and protamines (20) do, in fact, cause dissociation of receptor from catalytic proteins in protein kinases from other sources. We continued to use histone to assay column effluents for protein kinase activity but used casein to determine kinetic parameters for the three enzymes. The apparent $K_m$ values for kinases IA, IB, and II, respectively, were 2.5 (3.3), 3.3 (3.3), and 2.5 (3.3) mg of casein per ml, where the value in parenthesis represents the apparent $K_m$ in the presence of $6.7 \times 10^{-5} M$ cyclic AMP (Fig. 5). The plots were linear to a concentration of 0.25 mg casein per ml. The Michaelis constants for protein kinases are profoundly affected by salt concentration (16). The determinations reported here are for the purpose of demonstrating the probable identity of the catalytic subunits separated from the three kinase fractions.

Cyclic Nucleotides—The effects of various concentrations of cyclic AMP, 8-thiomethyl-cyclic AMP, 8-dimethylamino-cyclic AMP, and cyclic GMP on the sperm protein kinases are shown in Fig. 6. These nucleotides had generally the same effects on all three protein kinase forms. Half-maximal activation by cyclic AMP occurred in the $10^{-7} M$ range. As reported by others (21), the 8-methythio-cyclic AMP analogue appeared to be the most potent kinase activator. Cyclic GMP activated the protein kinases half-maximally at concentrations in the $10^{-6} M$ range. It appears that, in general, the more active the cyclic AMP analogue in activating the protein kinases at low concentrations, the greater the observed inhibition of kinase activity at higher concentrations of the analogue.

ATP—The apparent $K_m$ of ATP for kinase IA in the absence of cyclic AMP was about 4.2 $\mu$M (data not shown), while in the presence of $6.7 \times 10^{-5} M$ cyclic AMP it was 5.5 $\mu$M (Fig. 7). The $K_m$ of the catalytic unit of kinase IA was also 5.5 $\mu$M. The $K_m$ of kinase IB was about 5.5 $\mu$M and 3.5 $\mu$M in the absence or presence of cyclic AMP, respectively. The $K_m$ for kinase II was about 8.7 and 3.9 $\mu$M in the absence or presence of cyclic AMP, respectively. The apparent $K_m$ values of ATP in the presence of 7.5 mM Mg++, therefore, appear not to be different for the three protein kinases, nor to be altered by addition of cyclic AMP.

Cross-combination—Since R-1 was isolated free of contaminating catalytic activity, it was of interest to determine whether R-1 would cross-react with the catalytic units of the other pro-
tein kinases. Isolated catalytic subunits C-IA, C-II, and C-IB were successfully combined with R-I, using the inhibition of catalytic activity as evidence of combination (Table II). Addition of $6.7 \times 10^{-4} \text{M}$ cyclic AMP partially relieved the inhibition. Since inhibition was not completely relieved by cyclic AMP, this may indicate over-saturation by R-I in the system. Cross-combination was possible with the C-II fraction, despite the presence of R-2, due to failure of R-2 to recombine with C-II.

**DISCUSSION**

Reimann et al. (16) partially purified and studied the characteristics of rabbit skeletal muscle cyclic AMP-dependent protein kinases. They found two peaks of kinase activity after protein elution from DEAE-cellulose with a linear salt gradient, but they could find no appreciable kinetic differences between the two enzyme forms. Later in their work, they discovered that Peak I from DEAE-cellulose actually migrated as two peaks on sucrose density gradient centrifugation. The kinases isolated from bovine epididymal sperm react similarly when subjected to ion exchange chromatography. After a linear salt gradient elution, two peaks of kinase activity which co-elute with cyclic AMP-binding activity are located. Gel filtration of Peak I most often results in the separation of two peaks of different molecular weights (120,000 and 56,000). In one experiment, however, only the large molecular weight form (120,000) of the kinase was eluted from Sephadex G-100. Rubin et al. (22) have found only one large molecular weight form of the cyclic AMP-dependent protein kinase in heart muscle. With storage, this molecule will dissociate into smaller molecular weight fragments. The apparent subunit structure of the molecule is 2 catalytic units per 1 regulatory unit.

Our demonstration of multiple regulatory proteins in bovine epididymal sperm is not a unique observation. Corbin and Brostrom (23) working with skeletal muscle reported that two regulatory proteins could be separated from the kinase I eluted from DEAE-cellulose. The existence of the several regulatory proteins in three molecular weight forms of about 78,000, 35,000 to 40,000, and 17,000 to 18,000 suggest the possibility of a tetramer, dimer, monomer relationship between the cyclic AMP-binding proteins. However, for reasons described in the section on molecular weights, it seems more likely that proteolytic activity may be responsible for the smallest fraction. Determination of the natural protein substrates for the cyclic AMP-dependent protein kinases of sperm remains to be elucidated. It has been suggested by Hoskins and Stephens (24) that the stimulatory effect of cyclic AMP on sperm cell motility is mediated through the cyclic AMP-dependent protein kinases. Although this may prove to be true, no evidence yet exists to support this hypothesis. It is interesting that bovine spermatozoa contain neither detectable glycogen reserves (25) nor the enzyme phosphorylase (26). Sperm also lack the ability to synthesize new protein (25). The elimination of these two sites of cyclic AMP action makes bovine epididymal sperm a simpler cell system to study. Since it has been demonstrated that protein kinases phosphorylate neurotubule protein from brain (27), isolation of microtubule protein from sperm tails with estimation of phosphorylation rates in the presence or absence of cyclic AMP would be of interest. The observation of Yokota and Gots (9) that bacteria lacking the adenylate cyclase fail to synthesize flagella in the absence of cyclic AMP indicates effects of the nucleotide on microtubule protein.

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