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A CYCLIC AMP-STIMULATED PROTEIN KINASE FROM AMPHIBIAN OVARY AND OOCYTES

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SUMMARY

1. Cyclic AMP-stimulated protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) activity was found in extracts and purified preparations of ovary, oocytes, and liver of *Xenopus laevis*.

2. Phosphorylation of protein proceeded at a linear rate for at least 30 min regardless of substrate.

3. At low concentrations (0.2 mg/ml), histone readily served as a substrate for the enzyme, whereas casein and phosvitin did not.

4. Histone kinase activity was promoted by Mg^{2+} and Co^{2+} and was inhibited by Ca^{2+}, Cu^{2+}, and *p*-hydroxymercuriphenyl sulfonate.

5. At high concentrations (3.0 mg/ml), phosvitin served as a good substrate for a cyclic AMP-independent phosphorylation catalyzed by the same purified preparations that displayed cyclic AMP-stimulated histone kinase activity. The relationship between the two catalytic activities remains to be determined.

INTRODUCTION

Previous work[1] has documented that extracts and purified preparations derived from amphibian ovary and eggs promote the transfer of phosphate from the terminal position of ATP to certain proteins. Casein, lipovitellin, and particularly phosvitin served as the most effective substrates tested in the presence of the ovarian protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37). The suggestion was made at the time, therefore, that the phosphorylation of yolk proteins may be an important mechanism in the formation of yolk inclusions within the oocyte. A more recent biochemical study[2] on isolated oocytes undergoing yolk formation would seem to preclude this possibility, however, since the yolk proteins (lipovitellin and phosvitin) did not appear to be labeled up to 48 h after $^{32}P_i$ was supplied to the oocytes. Other unidentified proteins did become labeled, however, providing evidence for endogenous protein

phosphorylation. Because of these findings, we have initiated a new study to explore the significance of protein kinase in the amphibian oocyte.

Evidence for the presence of a variety of protein kinases stimulated by adenosine 3′:5′-monophosphate (cyclic AMP) has accumulated over the past few years[3]. These data suggest a common mechanism whereby cyclic AMP-stimulated protein kinases act as mediators of the many known and diverse effects of the simple molecule cyclic AMP. As an initial consideration, therefore, an investigation was undertaken to determine whether protein kinase activity in the ovary and oocytes of *Xenopus laevis* is promoted by the presence of cyclic AMP.

EXPERIMENTAL

Animals and chemicals

The procedures for the care and maintenance of animals and for anesthetization prior to ovariectomy have been described previously[4]. All animals were injected with 1000 units of human chorionic gonadotropin at least once within the week prior to use in order to ensure that the remaining oocytes were actively growing. Oocytes essentially free of follicle and other ovarian cells were isolated from the ovary by collagenase treatment[5], and the largest (1.0–1.2 mm diameter) were selected for the preparation of enzyme.

Purchased chemicals were from Sigma Chemical Corp., including casein ("α-casein") and calf thymus histone ("Type II-A"). Phosvitin (9.7% P) was prepared from *X. laevis* ovary by $(NH_4)_2SO_4$ precipitation and subsequent lyophilization[6]. High specific activity [γ-^{32}P]ATP was prepared by a modification of a previously described procedure[1] whereby unlabeled phosphate was omitted from the generation mixture (which contained 0.5 mCi carrier-free $^{32}P_i$ per ml), the ADP concentration was reduced to 4.6 μmoles/ml, and ATP was added to a concentration of 0.323 μmole/ml. After a 4-h incubation period at 20 °C, the reaction mixture was placed on a DEAE-cellulose column (30 cm × 1.9 cm) previously equilibrated with 1000 ml 0.25 M NH_4HCO_3 (pH 8.6) and 200 ml distilled water. A 1000-ml linear gradient ranging from 0.01 to 0.25 M NH_4HCO_3 (pH 8.6) was then passed through the column. Uncontaminated [γ-^{32}P]ATP, with better than 99% of the label in the γ-position, was eluted at a concentration of 0.17–0.19 M NH_4HCO_3 and collected free of solvent by repeated flash evaporation. It generally contained $1 \cdot 10^6$–$2 \cdot 10^6$ cpm/nmole.

Purification procedures

All operations were performed at 5 °C. For our initial survey, enzyme preparations were made from ovary and liver by the procedure of Kuo and Greengard[7] (extraction with 4 mM EDTA (pH 7.0); removal of insoluble material at pH 4.8; and, after neutralization, collection of the material precipitated by the addition of $(NH_4)_2SO_4$ to a concentration of 0.33 g/ml). A subsequent purification procedure was developed whereby ovarian tissue or oocytes were initially dispersed in two volumes of solution containing 250 mM sucrose, 10 mM potassium phosphate, and 4 mM EDTA (pH 7.0) by means of a glass homogenizer. The resulting homogenates were filtered through gauze and centrifuged at 16 000 × *g* for 30 min. Solid $(NH_4)_2SO_4$ was gradually added to the supernatants with stirring to a final concentration of 0.200 g/ml. After the $(NH_4)_2SO_4$ was dissolved, the mixtures were stirred for an additional 30 min and were

then centrifuged at 10 000 × *g* for 20 min. Additional $(NH_4)_2SO_4$ was added to the supernatants to provide a final concentration of 0.350 g/ml, and the equilibrated mixtures were centrifuged again. For chromatography, the final $(NH_4)_2SO_4$ precipitates were taken up in and thoroughly dialyzed against 5 mM potassium phosphate–2 mM EDTA (pH 7.0). After the protein concentration was determined, 275 mg of protein was added to a 30 cm × 1.9 cm DEAE-cellulose column equilibrated with 5 mM potassium phosphate–2 mM EDTA. Gradient elution was performed with a three-bottle gradient apparatus, the first two bottles each containing 335 ml 5 mM potassium phosphate–2 mM EDTA (pH 7.0) and the final bottle containing 330 ml 500 mM potassium phosphate–2 mM EDTA (pH 7.0). All chromatographic procedures were performed essentially as described previously[8].

Standard assay for protein kinase

Enzymatic preparations were tested for protein kinase activity by first dialyzing the preparation against 5 mM potassium phosphate–2 mM EDTA (pH 7.0). The protein concentration was then determined and adjusted to 200 μg/ml with fresh buffer. The adjusted enzyme solution was then added to the other components of the incubation mixture in an ice bath so that the final concentrations of ingredients were (per ml): 60 μg enzyme, 10 μmoles NaF, 1 μmole aminophylline, 0.3 μmole ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N',N'* tetraacetic acid (EGTA), 10 μmoles magnesium acetate, 50 μmoles glycerophosphate buffer (pH 6.5), 2.5 nmoles [γ-^{32}P]ATP, protein substrate (variable), either lacking or containing 5 nmoles cyclic AMP[7]. The reaction mixture was preincubated at 0 °C for 5 min and then transferred (at zero time) to 30 °C. Specific activity is defined as pmoles phosphate transferred to substrate protein per min/mg enzyme protein and is corrected for the phosphorylation of endogenous substrate (generally 0.04–0.08 pmole·min^{-1}·mg^{-1}).

Determination of total protein and $^{32}P_i$ incorporation into protein

Protein was determined by a modification of the method of Henry *et al.*[9], whereby 2.0 ml 1 M NaOH and then 0.5 ml biuret reagent were added to 0.5 ml protein solution. Absorbance was measured after 15 min at 545 nm, using bovine serum albumin as a standard.

Incorporation of ^{32}P into protein was measured by placing 100 μl incubation mixture onto filter paper discs (Whatman 3MM, 2.3 cm diameter) and immediately placing the disc into 10% trichloroacetic acid–5 mM NaH_2PO_4. 30 min after the last disc was added, they were transferred through five changes of 5% trichloroacetic acid–5 mM NaH_2PO_4 (15 min each), two changes of ethanol–ether (3:1, v/v; 30 min each), and two changes of ether (30 min each). The discs were then dried and counted in a Beckman Lowbeta II planchet counter. All counts were corrected to a [γ-^{32}P]ATP specific activity of $2 \cdot 10^6$ cpm/nmole.

RESULTS AND DISCUSSION

Preparations according to Kuo and Greengard

Kuo and Greengard[7] recently found evidence for the presence of cyclic AMP-stimulated protein kinases in preparations from different tissues of a variety of species, using both histone and casein as substrate. In preparations from mammalian

tissues, histone kinase activity was found to be more effectively promoted by cyclic AMP than casein kinase activity, whereas the reverse was true of nonmammalian tissues. We essentially followed the isolation procedures of Kuo and Greengard in order to obtain corresponding preparations from the ovary of *X. laevis*. However, we modified the assay methods by including a standardized preincubation period and by collecting multiple samples of the reaction mixture on discs in order to assure a linear reaction rate for at least 30 min.

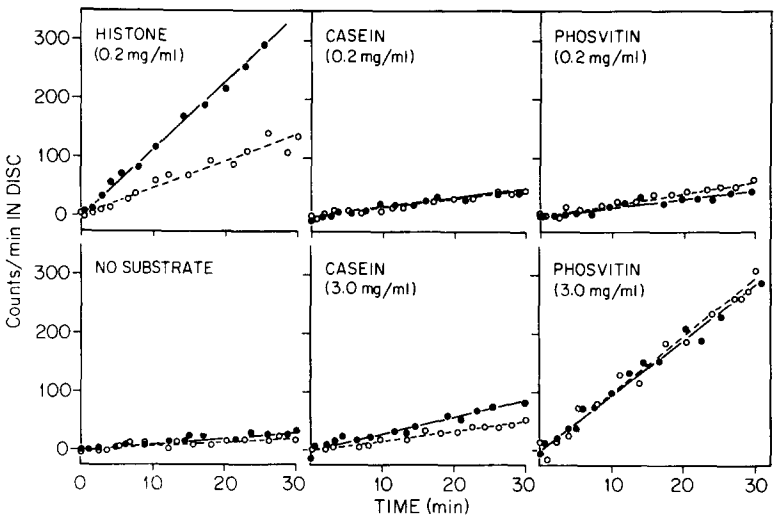


Fig. 1. Protein phosphorylation by an ovarian preparation in the presence of a variety of substrate conditions. Each protein substrate and the no-substrate control were assayed in the absence (○---○) and in the presence (●—●) of 5 μM cyclic AMP. The lines represent the least-squares fit to the experimental points.

Progress curves of protein phosphorylation found for a typical ovarian preparation (Fig. 1) indicate essentially linear kinetics regardless of the substrate used and whether cyclic AMP was present or not. The slope of the least-squares fit to the experimental points was therefore taken as a measure of protein kinase activity. The activity found for endogenous (no added) substrate was then subtracted from the values found in the presence of added substrates. The resulting data (Table I) indicate that when histone was used as a substrate there was a definite increase in the incorporation of radioisotope into protein in the presence of cyclic AMP. When casein and phosvitin were used as substrates in the same concentration as the histone (0.2 mg/ml), they were found to be relatively poor phosphate acceptors, with little evidence of stimulation by cyclic AMP.

Previous reports[10–12] have shown that cyclic AMP-stimulated protein kinases vary not only in their ability to phosphorylate different substrates but also in their sensitivities to different concentrations of substrate. Preparations derived from fish and from several invertebrates, for instance, promoted a greater cyclic AMP-stimulated phosphorylation of casein than histone when the former was present at a concentration of 3.0 mg/ml[7]. Yet even at this higher concentration, casein proved to be a relatively poor substrate for the ovarian protein kinase. Phosvitin, on the other hand,

TABLE I

EFFECT OF CYCLIC AMP ON ^{32}P INCORPORATION INTO DIFFERENT PROTEIN ACCEPTORS IN THE PRESENCE OF PROTEIN KINASE PREPARATIONS FROM THE OVARY AND LIVER OF *Xenopus laevis*

Tissue	*Substrate*	*Substrate concn (mg/ml)*	*Specific activity* (pmoles·min^{-1}·mg^{-1})*	
			Without cyclic AMP	*With cyclic AMP*
Ovary	Histone	0.2	0.33	0.78
	Casein	0.2	0.06	0.04
	Phosvitin	0.2	0.12	0.04
	Casein	3.0	0.08	0.15
	Phosvitin	3.0	0.76	0.70
Liver	Histone	0.2	0.17	2.00
	Casein	0.2	0.17	0.17
	Phosvitin	0.2	0.15	0.17
	Casein	3.0	0.07	0.29
	Phosvitin	3.0	0.83	1.00

* All data have been corrected for protein kinase activity which occurred in the absence of protein substrate.

also tested at a concentration of 3.0 mg/ml, promoted a rate of incorporation approximately equal to that found for histone at 0.2 mg/ml in the presence of cyclic AMP. However, the higher rate of incorporation into 3.0 mg phosvitin/ml appeared to be independent of cyclic AMP.

Enzyme preparations from *X. laevis* liver were made in a manner similar to those from the ovary and were assayed for protein kinase activity for comparison. The results (Table I) showed essentially the same pattern of activity as found for the ovarian preparations: stimulation by cyclic AMP when histone was used as the substrate, and significant phosphorylating activity when phosvitin was present at a concentration of 3.0 mg/ml with or without cyclic AMP.

Further purification and characterization of protein kinase from the ovary and oocytes

An alternate method of enzyme purification was devised which is more appropriate for isolating protein kinases from amphibian ovary. Fresh ovary was thoroughly homogenized in a buffered sucrose solution and centrifuged for 30 min at 16 000 × *g*. The resulting supernatant was collected and subjected to $(NH_4)_2SO_4$ fractionation. Each fraction was then assayed both for total protein and for specific activity with and without cyclic AMP. The resulting data (Fig. 2) indicate that considerable protein was precipitated as the $(NH_4)_2SO_4$ concentration was increased from 0.225 to 0.325 g/ml and that the specific activity of cyclic AMP-stimulated protein kinase was simultaneously augmented over this range. Material precipitating within a broad $(NH_4)_2SO_4$ concentration range of 0.200–0.350 g/ml was therefore used for subsequent investigation. Typical activity data (Table II) found for several such $(NH_4)_2SO_4$ preparations from ovary and isolated oocytes (1.0–1.2 mm diameter) indicate that (1) the specific activities are comparable to those of the Kuo and Greengard preparations (Table I), even though an acid precipitation step was omitted, and (2) the specific enzymatic activity of the oocyte preparation is comparable to that of ovary preparations.

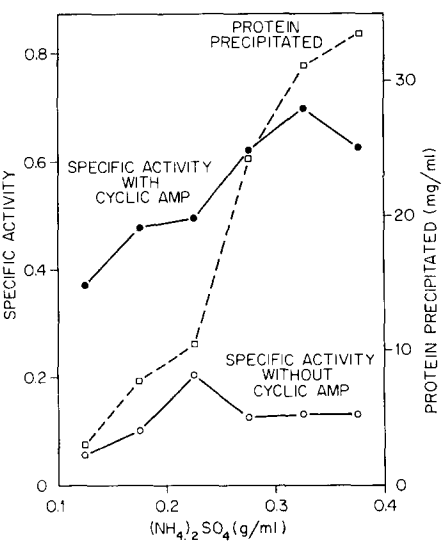
For further purification, the $(NH_4)_2SO_4$ preparations were subjected to DEAE-

TABLE II

PROTEIN KINASE ACTIVITY IN $(NH_4)_2SO_4$ FRACTIONS FROM OVARY AND OOCYTES

Method of extraction	*Specific activity** *(pmoles·min^{-1}·mg^{-1})*	
	Without cyclic AMP	*With cyclic AMP*
Ovary, Preparation 1	0.14	0.64
Ovary, Preparation 2	0.68	1.68
Oocytes (1.0–1.2 mm diameter)	0.56	1.17

* All data have been corrected for protein kinase activity which occurred in the absence of substrate (histone, 0.2 mg/ml).



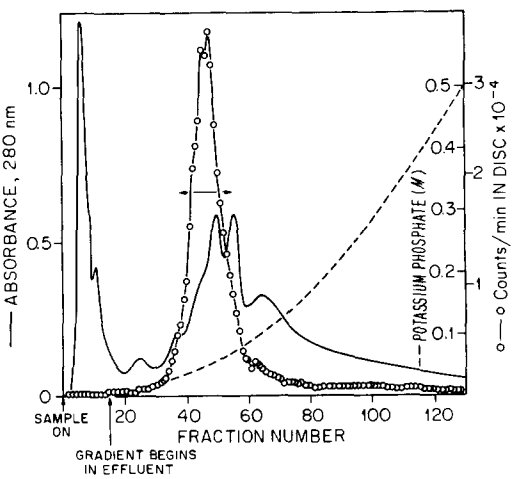


Fig. 2. The total protein precipitated at various $(NH_4)_2SO_4$ concentrations and the specific activity of each precipitate as determined in the absence (○—○) or in the presence (●—●) of cyclic AMP. An ovarian homogenate was centrifuged, the supernatant was divided into several batches, and to each batch solid $(NH_4)_2SO_4$ was added slowly over a 45-min period to the appropriate concentration at 5 °C. The mixtures were stirred an additional 30 min and then centrifuged at 10 000 × *g* for 20 min. The total protein (per ml original homogenate) and specific enzyme activity were determined for each precipitate. All data have been corrected for activity which occurred in the absence of substrate (histone, 0.2 mg/ml).

Fig. 3. Chromatography of an $(NH_4)_2SO_4$ fraction (0.200–0.350 g/ml) on DEAE-cellulose. Each eluant fraction contained 9.7 ml and was assayed for protein kinase activity by adding 50 μl to 150 μl reaction mixture at 0 °C. The final composition of the reaction mixture was the same as the standard assay mixture (including 0.2 mg histone and 5 nmoles cyclic AMP per ml), except that the protein content was variable and the [γ-^{32}P]ATP concentration was 5 nmoles/ml. After 45 min at 30 °C, 100 μl incubation mixture was placed on a disc, processed in the usual manner, and counted. No corrections were made for activity which occurred in the absence of protein substrate.

TABLE III

PROTEIN KINASE ACTIVITY IN CHROMATOGRAPHED PREPARATION UNDER VARIOUS CONDITIONS

Incubation conditions	*Specific activity* (pmoles·min^{-1}·mg^{-1})*	
	Without cyclic AMP	*With cyclic AMP*
Complete system, unfrozen enzyme	5.51	8.02
Complete system, frozen and thawed enzyme	3.72	5.21
Same, lacking Mg^{2+}	0.02	0.01
Same, 10 mM Co^{2+} replacing Mg^{2+}	4.48	5.10
Same, 10 mM Mn^{2+} replacing Mg^{2+}	1.63	2.05
Same, 10 mM Cu^{2+} replacing Mg^{2+}	0.01	0.02
Same, 10 mM Ca^{2+} replacing Mg^{2+}	0.06	0.03
Same, 10 mM Cu^{2+} added	—	0.28
Same, 10 mM Ca^{2+} added	—	0.82
Same, 1 mM *p*-hydroxymercuriphenyl sulfonate added	—	0.10

* All data have been corrected for protein kinase activity which occurred in the absence of substrate (histone, 0.2 mg/ml).

cellulose chromatography, using a continuous gradient ranging from 5 to 500 mM potassium phosphate (pH 7.0). Fractions were collected and assayed for protein kinase in the presence of cyclic AMP. As shown in Fig. 3, a single peak of activity was obtained. The active fractions (shown by an arrow in Fig. 3) were pooled, concentrated by ultrafiltration[8], and assayed in the usual manner, both in the presence and in the absence of cyclic AMP. The resulting data (Table III) indicate that although the specific activity of the enzyme was increased by a factor of about 10, the stimulation by cyclic AMP was greatly reduced. The same loss of cyclic AMP stimulation was found when nonchromatographed preparations were assayed after being slowly frozen to −20 °C and thawed. Finally, when the chromatographed protein kinase preparation was frozen, subsequently thawed, and assayed again for activity, a decrease in the specific activity was noted relative to that found in the chromatographed but unfrozen preparation (Table III). These observations would thus argue against storage of the enzyme in the frozen state.

A preliminary survey on the effects of metal ions on catalytic activity was performed, and the results corresponded to the findings of other investigators on cyclic AMP-stimulated protein kinases in a variety of cells (Table III)[11–13]. Mg^{2+} promoted optimal activity as did Co^{2+}, while Mn^{2+} promoted moderate activity. In contrast, no activity was observed when either Ca^{2+} or Cu^{2+} was substituted for Mg^{2+}. Ca^{2+} and Cu^{2+} were also added to standard assay mixtures containing Mg^{2+}, and in both cases protein kinase activity was severely inhibited. The sulfhydryl reagent *p*-hydroxymercuriphenyl sulfonate also severely inhibited enzymatic activity when added to the standard assay mixture, suggesting that a sulfhydryl group is an essential component of the catalytic site.

Conclusions and interpretation

The data obtained in this preliminary study indicate that a cyclic AMP-stimulated protein kinase is present in the ovary and specifically in the oocytes of *X. laevis*,

and its properties appear similar to the enzyme activity present in homologous liver preparations as well as those from other organisms. Activity was measured by a standardized procedure[7], however, and no attempt was made to characterize the ovarian enzyme more specifically with regard to the relative effectiveness of various concentrations of cyclic AMP or other cyclic nucleotides. Histone was found to be a good substrate for the enzyme when present at a concentration of 0.2 mg/ml, whereas casein and phosvitin were not. If the concentration of phosvitin was increased 15-fold, however, it was also found to be a good substrate, although phosphorylation in this case was not cyclic AMP-dependent. Stimulation of histone kinase activity by cyclic AMP was also less marked in preparations that had been chromatographed or frozen and thawed.

Several investigators[11,13] working with mammalian tissues have proposed that cyclic AMP-stimulated protein kinases are comprised of a regulatory (inhibitory) subunit which binds cyclic AMP, and a catalytic subunit. After binding cyclic AMP, the regulatory subunit dissociates from the catalytic subunit, which in turn becomes free to promote the phosphorylation of a suitable protein substrate. If a similar complex exists in the ovary or oocyte, the loss of cyclic AMP dependency which we have observed after freezing and thawing may simply be caused by a dissociation of subunits. Certain protein substrates have also been found recently to promote the dissociation of protein kinases[14]. Phosvitin may thus act in a similar way to dissociate the regulatory and catalytic subunits of ovarian protein kinase when present in high concentration, because of its high surface charge. Alternatively, the cyclic AMP-stimulated histone kinase and the cyclic AMP-independent phosvitin kinase may represent two different enzymes, the latter being similar or identical to the protein kinase previously found in the ovary and eggs of *Rana pipiens*[1].

It is uncertain whether histone represents the natural substrate for cyclic AMP-stimulated protein kinase in the oocyte. If the enzyme has a functional role in the oocyte, then the natural substrates may rather be those nonhistone-like but as yet unidentified proteins found to be labeled when isolated growing oocytes were incubated in the presence of $^{32}P_i$ (ref. 2). On the other hand, many substances, including enzymes, accrue within developing amphibian oocytes and are subsequently utilized during embryogenesis. If this is the case for the cyclic AMP-stimulated protein kinase of the oocyte, then the natural substrates have yet to be indicated and may very well include histone.

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