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GUANOSINE 3′:5′ MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM BOVINE ADRENAL CORTEX: PARTIAL PURIFICATION AND CHARACTERIZATION

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SUMMARY

Cyclic GMP-dependent protein kinase has been partially purified from bovine adrenal cortex. The enzyme was specifically activated by cyclic GMP at lower concentrations. The K_a for cyclic GMP was 9×10^{-8} M whereas that for cyclic AMP was 7×10^{-6} M. An optimum concentration of 75 mM magnesium was needed for maximal stimulation by cyclic GMP whereas 10 mM magnesium ion concentration was found to be optimal for the cyclic AMP-dependent protein kinase.

The enzyme was found to have a molecular weight of 225,000 based on gel filtration. When the enzyme was photoaffinity labeled with cyclic GMP, it again demonstrated the molecular weight of 225,000. This indicated that the enzyme was not dissociated into regulatory and catalytic components by cyclic GMP. The protein was composed of two polypeptides having the same molecular weight of 110,000 as shown by SDS-PAGE, and thus it appears to consist of two subunits of equal size.

This study thus provides the direct evidence for the presence of cyclic GMP-dependent protein kinase in adrenal cortex and lends additional support for the molecular model proposed earlier from our laboratory postulating a crucial role for this protein in adrenal steriodogenesis [4].

INTRODUCTION

Previous studies with isolated rat adrenal cells from this laboratory have demonstrated the important physiological mediatory role of cyclic GMP in ACTH-induced steroidogenesis [1–4] (for a review see ref. [5]). A correlation between cyclic GMP-activated steroidogenesis and endogenous protein kinase activity in the fasciculata cell has been demonstrated [2]. These studies have provided indirect support of the proposal that the translational control step of ACTH-induced steroidogenesis mediated by cyclic GMP is the entry of the cytoplasmic cholesterol pool into the mitochondria via the activation of cyclic GMP-dependent protein kinase [6]. To date, however, no direct demonstration of the presence of cyclic GMP-dependent protein kinase in adrenal cortex has been reported. It is therefore obvious that, despite a strong indirect evidence favoring mediation by cyclic GMP-dependent protein kinase in cyclic GMP-activated adrenal steroidogenesis, the proposition [4] would remain speculative until direct evidence for the existence of a specific cyclic GMP-dependent protein kinase is demonstrated in the adrenal cortex. This paper describes the presence of such a protein and thus provides an additional evidence in support of our previous model [4] which depicts a pivotal role of cyclic GMP-dependent protein kinase in ACTH-induced steroidogenesis. We describe here the partial purification and characterization of the cyclic GMP-dependent protein kinase from the bovine adrenal cortex. A preliminary account of these studies has been provided elsewhere [7].

The trivial names and abbreviations used are: ACTH, adrenocorticotropic hormone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis (2-aminoethyl)-N,N′-tetraacetic acid; TME, 20 mM Tris HCl buffer (pH 7.5) containing 5 mM β-mercaptoethanol; and 0.2 mM EDTA; TMGE, TME buffer containing 5% glycerol; TME-G_{50}, TME buffer containing 50% glyocerol; TCA, trichloroacetic acid.

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EXPERIMENTAL PROCEDURES

Materials. Bovine adrenal glands were obtained fresh from the slaughter house. Cyclic nucleotides, calf thymus histone (Type IIA) and Tris-base were from Sigma Chemical Co; bovine serum albumin (mixture of dimer and trimer) was from Miles Laboratories, Inc.; bovine serum albumin (monomer), aldolase, ovalbumin and ribonuclease were from Pharmacia Fine Chemicals, Inc., and *Escherichia coli* RNA-polymerase and Polymin P were gifts from Dr. R. R. Burgess, McArdle Laboratories, Madison, Wisconsin. Ammonium sulfate (enzyme grade) was from Schwarz/Mann; DEAE-cellulose (DE52) and GF/C glass-fiber microfilters were from Whatman Biochemicals Ltd; Ultragel ACA-34 was from LKB Instruments Inc.; Reagents for polyacrylamide gel electrophoresis were from BioRad Laboratories and toluene (scintillation grade) was from Fisher Scientific Comp.

[γ-^{32}P]ATP (3000 Ci mmol) was from Amersham Corp. and cyclic [^{3}H]-GMP (40 Ci/mmol), cyclic [H^3]AMP (40 Ci/mmol) and Omnifluor Scintillator were from New England Nuclear. All other chemicals were reagent grade and obtained commercially.

Cyclic nucleotide binding was assayed by a modification [8] of the Gilman procedure [9]. A 50 μl aliquot of enzyme was incubated at 0°C for 60 min in 50 mM sodium acetate buffer (pH 4.0), 2 mM EDTA and 3 pmol of cyclic [^{3}H]GMP or cyclic [^{3}H]AMP in an incubation volume of 0.1 ml. The reaction was initiated by the addition of the enzyme and terminated with 2 ml of ice cold 70% saturated ammonium sulfate. After 20 min the precipitate was collected on GF/C filters, washed thrice with 25% saturated ammonium sulfate, and the filters were dried and counted for radioactivity in 5 ml of Omnifluor cocktail in toluene (4g/l).

Photoaffinity labeling of cyclic nucleotide binding proteins was carried out in TMGE buffer (pH 7.5). One millilitre of the DEAE-cellulose fraction (0.05–0.15 M NaCl) was preincubated at 0°C for 30 min with 30 pmol of cyclic [^{3}H]-nucleotides. This was followed by incubation in a spectrophotometric quartz cuvette at 0°C for 8 h in front of a mineralite UVSL-25 hand lamp [10]. The reaction mixture was analyzed by gel filtration and SDS-PAGE for determination of the molecular weight and subunit composition of the enzyme.

Protein kinase [11] was assayed at 37°C for 5 min in a 500 μl incubation volume containing 50 mM sodium glycerol-2-phosphate (pH 6.0), 10 mM magnesium acetate, 20 mM sodium fluoride, 0.3 mM EGTA, 500 μg histone and 200 μM [γ-^{32}P]-ATP (1 μCi). 100 μM cyclic GMP or cyclic AMP were added to determine the stimulation by these nucleotides. The reaction was started by the addition of 50 μl of enzyme solution and terminated with 2 ml of 10% TCA. The precipitate was collected after 20 min, dissolved in 0.5 ml of 0.5 M NaOH and reprecipitated in 10% TCA. The acid precipitable radioactivity was collected on GF/C filters, washed thrice with 10% TCA, dried and counted in Omnifluor cocktail. Changes in the magnesium acetate and cyclic nucleotide concentrations for different experiments are indicated in the figure legends.

Partial purification of cyclic GMP-dependent protein kinase-Bovine adrenal glands (800 g) were dissected into medulla and cortex and the cortical tissue was homogenized with 2 vol. of TME buffer (pH 7.5) in a Waring blender. The postmitochondrial supernatant obtained by centrifugation at 30,000 *g* for 45 min was subjected to Polymin P fractionation [1, 13]. A 10% Polymin P stock solution (pH 7.5) was added to a final concentration of 0.1%, the mixture was centrifuged, and to the supernatant more 10% Polymin P was added to a final concentration of 0.4%. After centrifugation, this precipitate contained all the kinase activity. It was dissolved in TME buffer and solid ammonium sulfaate was added to 50% saturation. The precipitate was collected, dissolved in TME buffer, and dialyzed overnight against TMGE buffer. The dialyzed fraction was applied to a 1500 ml DEAE-cellulose column previously equilibrated with TMGE buffer. The column was washed with 4.5 l TMGE + 0.05 M NaCl, followed by 4.5 l of TMGE + 0.15 M NaCl and finally with 3 l of TMGE + 0.5 M NaCl. Most of the cyclic GMP binding and kinase activity was found in the 0.15 M NaCl fraction. This was concentrated by ammonium sulfate precipitation (50% saturation), dialyzed overnight against TMGE, and finally dialyzed against TME-G_{50} + 0.1 M NaCl for storage at −20.° This enzyme preparation was used in all experiments unless otherwise indicated.

Samples analyzed by gel filtration were applied to a 130 ml column (1.6 × 65 cm) of Ultragel ACA-34 previously equilibrated with TMGE + 0.25 M NaCl. The resin was eluted at a flow rate of 15 ml/h with the same buffer and 1 ml fraction were collected. For determination of the molecular weight, the column was calibrated with bovine serum albumin (trimer, dimer and monomer), aldolase and ovalbumin. The void volume of the column was determined with blue dextran and the column volume with the free cyclic [^{3}H]-nucleotide in the analyzed samples.

SDS-PAGE was run as described by Laemmli[14] with the modifications described by Burgess and Jendrisak[12]. Photoaffinity labeled DEAE-cellulose and Ultragel ACA-34 fractions were TCA precipitated (10%) and dissolved in 500 μl SDS-sample buffer (pH 6.8) prior to electrophoresis. The gels were cut into 2 mm slices, and each slice was dissolved in 0.5 ml of 30% hydrogen peroxide and counted in 10 ml of Scintiverse cocktail. *E. coli* RNA-polymerase subunits, bovine serum albumin and RNase were used as molecular weight marker proteins. These gels were stained with 0.05% Coomassie Brilliant Blue R-250 and diffusion destained in 7.5% acetic acid [13].

RESULTS AND DISCUSSION

Several laboratories have isolated the cyclic GMP-dependent protein kinase from mammalian cerebellum and lung tissues [15–22]. These tissues are relatively rich in cyclic GMP content and cyclic GMP-dependent protein kinase. Although the isolation of crude adrenal cyclic GMP-binding protein has been described in a preliminary report [23], this is the first report on the isolation and characterization of the cyclic GMP-dependent protein kinase from bovine adrenal cortex.

Our initial studies with the adrenal gland indicated that the cyclic GMP-binding protein kinase is extremely unstable and is present in a small quantity as compared to the cyclic AMP-binding protein kinase. Thus our initial attempts to characterize this enzyme were frustrated by enzyme inactivation during the long intervals of time involved in various chromatographic procedures for isolation and purification.

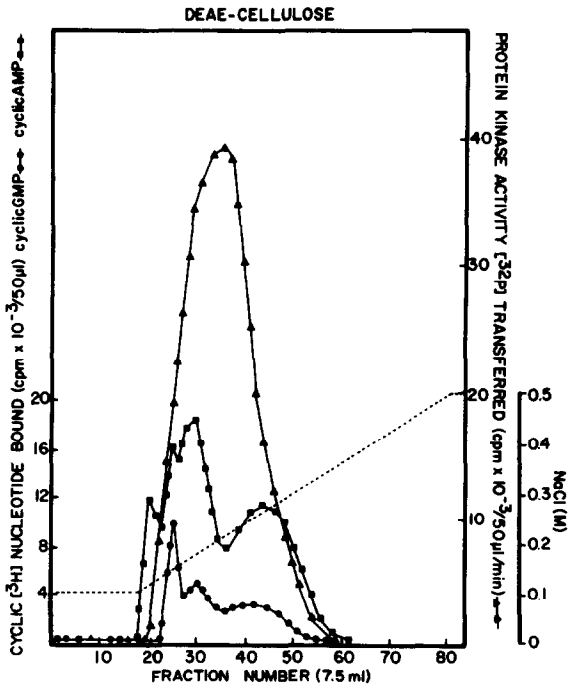
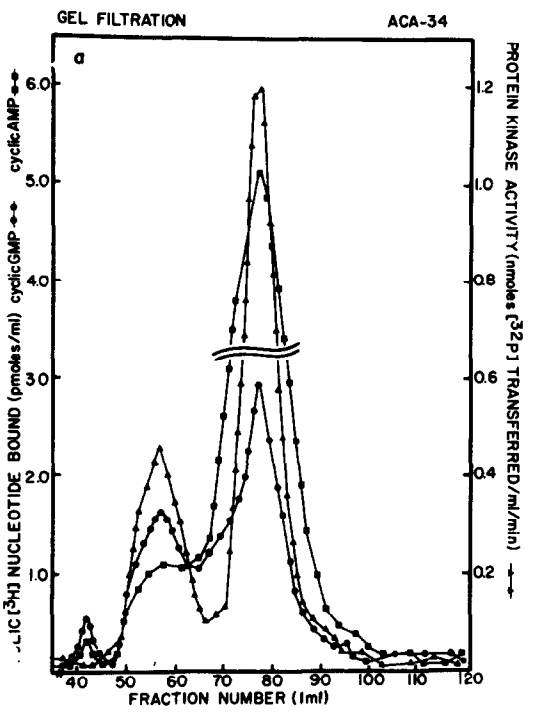


Fig. 1. DEAE-cellulose chromatography of cyclic GMP and cyclic AMP-dependent protein kinases from bovine adrenal cortex. The 150 ml column was eluted with a 500 ml linear concentration gradient of NaCl (0.1–0.5 M) in TMGE buffer at 75 ml/h and 7.5 ml fractions were collected. A 50 µl aliquot of each fraction was assayed for cyclic GMP ●—● and cyclic AMP ■—■ binding at pH 4 as described, and for kinase activity ▲—▲ at pH 6 in the presence of 100 µM cyclic GMP. These values have been corrected for kinase activity obtained in the absence of cyclic nucleotide. This is a typical gradient elution pattern under these conditions. Subsequent experiments used step elution for the isolation of cyclic GMP-binding protein kinase. The 1500 ml column was washed with 0.05 M NaCl in TMGE buffer to remove cyclic AMP binding protein devoid of cyclic GMP binding and kinase activity; the cyclic GMP-dependent protein kinase was then eluted with three column volumes of TMGE buffer containing 0.15 M NaCl. Most of the cyclic AMP-dependent protein kinase eluted at higher salt concentrations.

These problems led us to a combination of two techniques for demonstrating the presence of this cyclic GMP-binding protein kinase in the adrenal gland. We achieved partial purification of the enzyme by a series of steps involving fractionation by Polymin P, ammonium sulfate precipitation, and ion exchange chromatography on DEAE-cellulose (Fig. 1). The molecular weight was established by gel filtration. The subunit structure and the ultimate characterization of this protein was, however, greatly facilitated by the cyclic GMP photoaffinity probe covalently linked with the cyclic GMP-binding protein. It was found that there was some cross-reaction with the cyclic AMP-binding protein, but the specific activity of the label incorporated into the cyclic GMP-binding fraction was at least 4-fold higher (Figs. 2b and 3a).

The molecular weight of the enzyme is approximately 225,000 which is significantly higher than that reported for lung [16, 21, 23]. SDS-PAGE of photo-



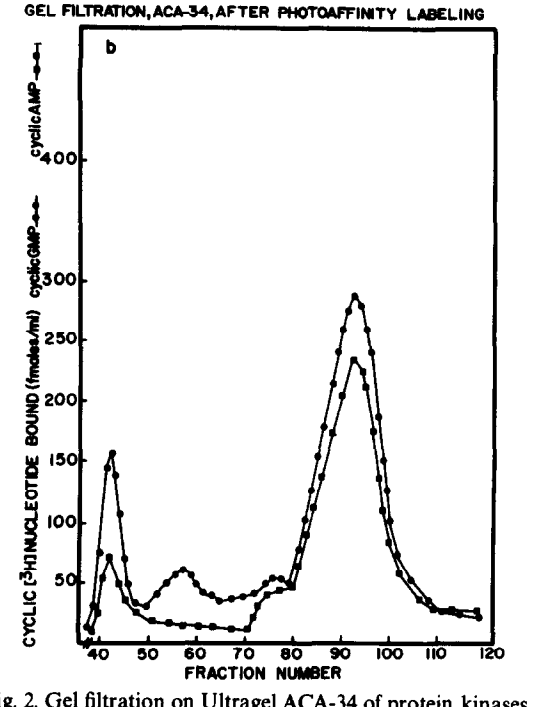


Fig. 2. Gel filtration on Ultragel ACA-34 of protein kinases after DEAE-cellulose chromatography. The 130 ml column was eluted at 15 ml/hr with TMGE buffer containing 0.25 M NaCl and 1 ml fractions were collected. a. Proteins eluted from DEAE-cellulose between 0.05 and 0.15 M NaCl were analyzed. A 50 µl aliquot of each fraction was assayed for cyclic GMP ●—● and cyclic AMP ■—■ binding and kinase activity ▲—▲. Fraction 57 yielded the cyclic GMP-binding protein kinase peak, whereas the cyclic AMP-binding protein kinase peak eluted at fraction 78. b. Identical DEAE-cellulose fractions were photoaffinity labeled with either cyclic [^{3}H]-GMP or cyclic [^{3}H]-AMP at pH 7.5 and 0°, prior to separation on Ultragel ACA-34. The 1 ml fractions were analyzed for radioactivity.

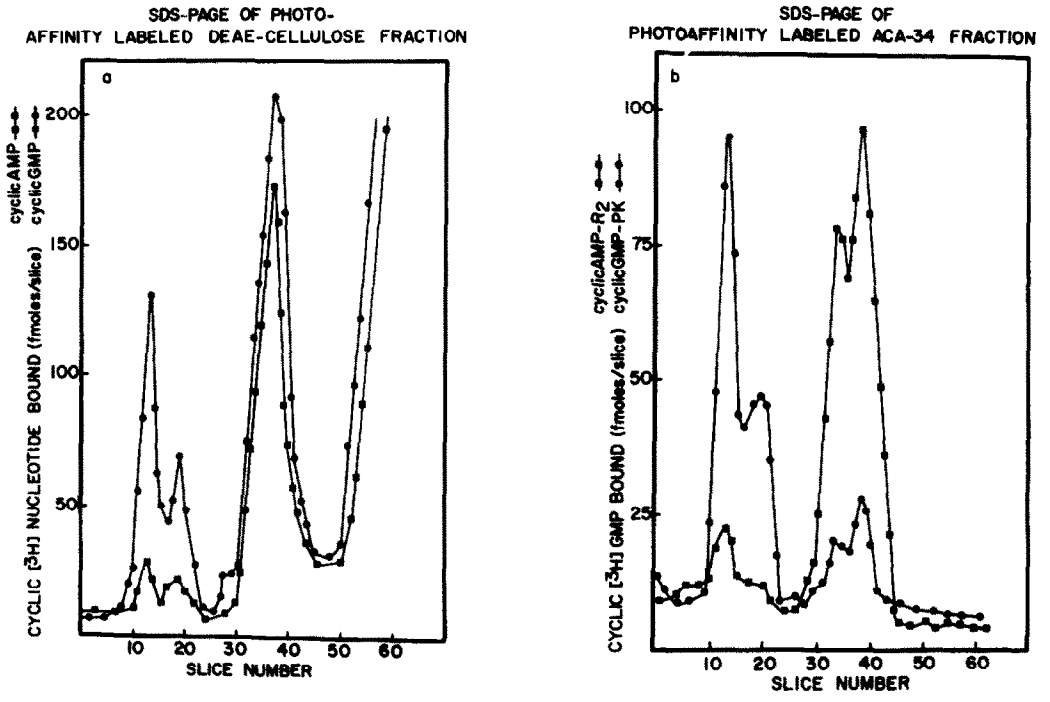


Fig. 3. SDS-PAGE of the photoaffinity labeled DEAE-cellulose fraction. a. The DEAE step elution fraction (0.05–0.15 M NaCl) was photoaffinity labeled with cyclic [^{3}H]-GMP ●—● or cyclic [^{3}H]-AMP ■—■ and subjected to electrophoresis on SDS-polyacrylamide gels. b. The same DEAE-cellulose fraction was photoaffinity labeled with cyclic [^{3}H]-GMP and passed through Ultragel ACA-34. Fraction 57 corresponding to the cyclic GMP-dependent protein kinase (cGMP-PK ●—●) and fraction 92 corresponding to the regulatory subunit dimer of the cyclic AMP-dependent protein kinase (cAMP-R_2 ■—■) were analyzed by SDS-PAGE.

affinity labeled cyclic GMP-binding protein revealed that only one type of subunit of 110,000 daltons exists in the preparation of cyclic GMP-dependent protein kinase (Fig. 3a). When the molecular weight of the photoaffinity labeled enzyme was determined by ACA-34 gel filtration, it was found to be 225,000 (Figs 2b and 4). The molecular weight of the native enzyme by gel filtration was also found to be 225,000 (Figs 2a and 4). The same results were obtained when the enzyme was first photoaffinity labeled with cyclic GMP and the 225,000 dalton peak obtained by gel filtration was subjected to SDS-PAGE. This yielded the labeled 110,000 dalton protein (Fig. 3b). Another minor cyclic GMP-binding peak of 82,000 daltons was also detected which could be a degraded product of the parent enzyme. Since the native enzyme binds

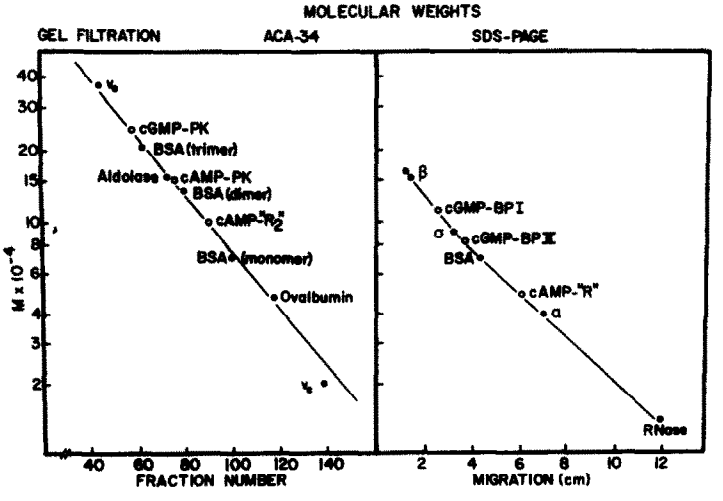


Fig. 4. Summary of molecular weights of cyclic GMP and cyclic AMP dependent protein kinases and their subunits obtained by gel filtration on Ultragel ACA-34 and SDS-PAGE.

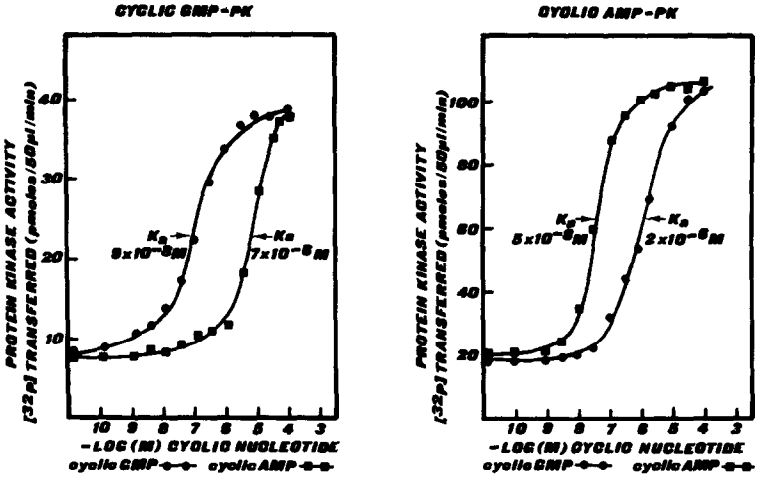


Fig. 5. Stimulation by cyclic GMP ●—● and cyclic AMP ■—■ of the two cyclic nucleotide-dependent protein kinase peaks obtained by gel filtration on ACA-34. Kinase activities of the cyclic GMP-dependent protein kinase (left) and cyclic AMP-dependent protein kinase (right) were assayed in the presence of 75 mM and 10 mM magnesium acetate, respectively. Other experimental conditions are as described.

cyclic GMP and catalyzes the phosphorylation of histone, the enzyme appears to be a dimer of the 110,000 dalton subunit. This also indicates that the cyclic GMP-dependent protein kinase is not dissociated by cyclic GMP to exhibit its catalytic activity. These results are in accord with the properties of the enzyme isolated from lung [20–22]. Figure 5 shows that the apparent activation K_a of the cyclic GMP-dependent protein kinase by cyclic GMP is 9×10^{-8} M. This value is 100-times lower than that obtained with cyclic AMP. These K_a values are somewhat higher than the published values for cyclic GMP-dependent protein kinase from lung where the K_a of 2×10^{-8} M has been found [18–20]. In accord with the findings observed with lung tissue, higher concentrations of magnesium ion (75 mM) were necessary for cyclic GMP-dependent kinase activity whereas cyclic AMP-dependent protein kinase required only 10 mM magnesium (Fig. 6). The isolation of the cyclic GMP-dependent enzyme as a holoenzyme by gel filtration using the cyclic GMP-photoaffinity labeled probe provides strong evidence against its dissociation into presumptive R and C subunits as proposed by some laboratories [24–26]. These results, however, are in accord with the findings of various other investigators [20–22].

Previous studies with isolated adrenal cells have demonstrated that physiological concentrations of ACTH raise the levels of cyclic GMP which in turn activates the endogenous protein kinase [1, 2] and the onset of steroidogenesis. The direct stimulatory effect of ACTH and cyclic GMP-activated steroidogenesis utilizing endogenous precursor, presumably cholesterol, is blocked by cycloheximide [29–31]. Indirect evidence has been provided that the cycloheximide-sensitive translational control of the hormone mediated by cyclic GMP is at the entry of cytoplasmic cholesterol into the mitochondria [6]. It has been further demonstrated that the cycloheximide-sensitive step is after the activation of protein kinase [2]. The

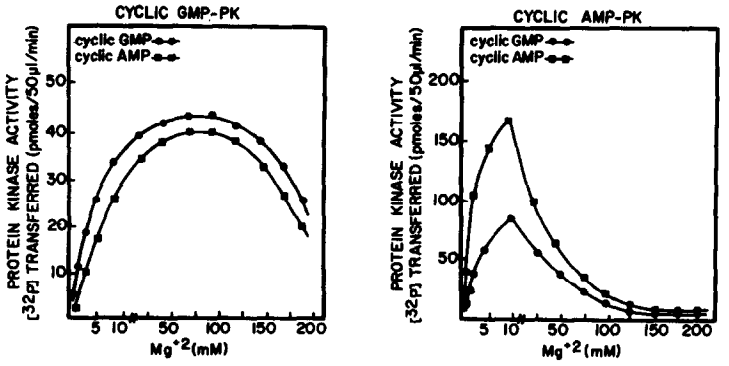


Fig. 6. Effect of varying concentrations of magnesium acetate on cyclic nucleotide-dependent protein kinase activity of enzymes obtained by gel filtration on ACA-34. The assay was performed with 50 µl aliquot under standard conditions in the presence of 10 µM cyclic GMP ●—● or cyclic AMP ■—■.

present results thus provide the direct evidence for the presence of cyclic GMP-dependent protein kinase in adrenal cortex and lends additional support for the molecular model proposed earlier from our laboratory postulating a crucial role for this protein in adrenal steroidogenesis [4].

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