may be an important mechanism of regulation of the Krebs cycle in *Neurospora crassa*.

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Cyclic Adenosine 3′,5′-Monophosphate Dependent Protein Kinase of Rat Leydig Cells: Physical Characteristics of Two Holoenzymes and Their Subunits†

E. J. Podesta, M. L. Dufau, and K. J. Catt*

ABSTRACT: The interstitial cells of the rat testis contain two forms of cAMP-dependent protein kinase which are activated in vitro by low concentrations (10^{-11} M) of human chorionic gonadotropin. The two cAMP-dependent holoenzymes of purified Leydig cells have been further characterized by cAMP binding and phosphokinase assay during ion-exchange chromatography, gel filtration, and sucrose density gradient centrifugation. Equilibrium binding studies performed with interstitial cell extracts and [^{3}H]cAMP revealed a single order of binding sites with high affinity for cAMP ($K_a = 10^9$ M^{-1}). After chromatography on DEAE-Sephadex and DEAE-cellulose, the two holoenzymes were identified by density gradient centrifugation as discrete peaks with sedimentation constants of 4.0 S and 6.2 S. From these values and the Stokes radii of 37.9 Å and 47.7 Å derived from gel filtration on Sephadex G-200, the estimated molecular weights of the holoenzymes were 59 600 and 116 400, respectively. The regulatory subunits of the protein kinase holoenzymes were also identified by cAMP binding analysis after gel filtration and sucrose density centrifugation, as 3.0S and 4.2S components with estimated molecular weights of 35 500 and 66 300. The molecular weight of the 2.9S catalytic subunit was estimated to be 33 000. The contributions of the 6.2S and 4.0S forms of the holoenzyme to the total cAMP-dependent protein kinase activity in Leydig cell extracts were approximately 70% and 30%, respectively. The main major 6.2S holoenzyme was eluted by 120 mM NaCl during chromatography on DEAE-cellulose at pH 7.4 and exhibited properties comparable with those of the type I protein kinase present in other tissues. However, the 4.0S holoenzyme eluted by 220 mM NaCl did not correspond to the type II holoenzyme of other tissues and appeared to be derived from the 6.2S holoenzyme. Selective or sequential activation of the individual forms of protein kinase during hormone stimulation could provide a mechanism for the expression of discrete biological responses in the Leydig cell.

Activation of cAMP[1]-dependent protein kinase by gonadotropic hormones has been recently demonstrated in collagenase-dispersed interstitial cells of the rat testis (Podesta et al., 1976a,b). Enzyme activity was stimulated by incubation of the isolated cells with low concentrations of luteinizing hormone (LH) and human chorionic gonadotropin (hCG). The extent of enzyme activation was correlated with the production of cAMP by the hormone-stimulated cells (Podesta et al., 1976a,b; Cooke & van der Kemp, 1976). The interstitial cell has been shown to contain two forms of cAMP-dependent protein phosphokinase, with sedimentation constants of about 6.5 S and 3.8 S. During incubation of isolated interstitial cells with trophic hormones, total protein kinase activity was stimulated in a dose-dependent manner, leading to the formation of a single 2.9S catalytic subunit. Conversion of the

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[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; LH, luteinizing hormone; hCG, human chorionic gonadotropin; BSA, bovine serum albumin.

holoenzymes to the catalytic subunit was demonstrable at physiological concentrations of gonadotropin, as low as 10^{-11} M (Podesta et al., 1976b).

The action of gonadotropins upon steroidogenesis in testis and ovary is believed to be mediated through cAMP, with subsequent activation of protein kinase and phosphorylation of regulatory proteins which control steroid biosynthesis. However, previous studies suggested that stimulation of testicular steroidogenesis by very low concentrations of hCG was not accompanied by a commensurate change in cAMP levels (Mendelson et al., 1975) or protein kinase activity (Podesta et al., 1976a). Recently, small changes in total protein kinase activity (Cooke et al., 1976; Conn et al., 1977, Dufau et al., 1977) and significant increases in cAMP binding to protein kinase (Dufau et al., 1976, 1977) have been observed during gonadotropin action in isolated Leydig cells. Further resolution of the role of the cAMP-protein kinase system in hormonal stimulation of steroidogenesis and other gonadotropin-induced responses could depend upon a more detailed clarification of the nature and activation properties of Leydig cell protein kinase. Since previous information about the properties of protein kinase in Leydig cells have been derived from studies in whole testes or interstitial cell preparations, it was of interest to analyze the interaction of cyclic AMP with the holoenzymes and regulatory subunits of the protein kinase from highly purified Leydig cells. These experiments have defined the physical and functional characteristics of the two holoenzymes, and have demonstrated the binding properties and physical features of the corresponding regulatory and catalytic subunits.

Materials and Methods

Reagents. [^{3}H]cAMP (23 Ci/mmol) and sucrose were purchased from Schwarz/Mann, Orangeburg, N.Y., and [^{32}P]ATP (30 Ci/mmol) was from ICN Pharmaceuticals Inc., Irving, Calif. ATP, cAMP, cGMP, and DEAE-cellulose were obtained from Sigma Chemical Co. Collagenase type I and histone subfraction F2b were purchased from Worthington Biochemical Corp., Freehold, N.J. DEAE-Sephadex A-50 was obtained from Pharmacia, cellulose filters, type HA 0.45 μm, were purchased from Millipore Corp., Bedford, Mass., and bovine serum albumin (BSA) from Metrix Corp. Metrizamide (Nyegaard) was supplied by the Accurate Chemical Co., Hicksville, N.Y. Purified human chorionic gonadotropin (10 000 IU/mg) was a gift of Dr. R. E. Canfield, Department of Medicine, Columbia University, New York, N.Y.

Preparation of Interstitial Cells and Purified Leydig Cells. Testes removed from adult (250–300 g) Sprague-Dawley rats were decapsulated and incubated at 37 °C in groups of four to six in 45-mL polyethylene tubes (Falcon) containing 10 mL of medium 199 with bovine serum albumin (1 mg/mL) and collagenase (0.25 mg/mL), with shaking at 150 cycles/min. The enzyme-dispersed interstitial cells were harvested after incubation with collagenase for 15 to 20 min (Mendelson et al., 1975; Dufau & Catt, 1975), and purified by Metrizamide gradients as previously described (Conn et al., 1977). For isolation of Leydig cells, 10^8 interstitial cells in 2 mL of medium 199 containing 0.1% BSA were applied to a 40-mL gradient of 0–80% Metrizamide prepared in Medium 199/BSA, and centrifuged at $3300g_{av}$ for 5 min. The Leydig cell fraction was removed by aspiration, diluted in medium 199, and collected by centrifugation at $225g_{av}$ for 10 min. This procedure provided purified Leydig cells of about 90% homogeneity which retained their characteristic morphological and biochemical properties. Incubation of purified Leydig cells with trophic hormone and assay of protein kinase activity were performed as previously described (Podesta et al., 1976a,b). The protein kinase assay was based upon the method of Reddi et al. (1971) with minor modifications, employing histone F2b as substrate.

Ion-Exchange Chromatography. Cell suspensions were sonicated with the microtip of a Bransome cell disruptor Model 401 for 15 s and then fractionated by stepwise gradient elution from DEAE-Sephadex A-50, or by continuous gradient elution from DEAE-cellulose. For the DEAE-Sephadex procedure, 3-mL aliquots of a slurry of DEAE-Sephadex A-50 in distilled water (equivalent to 30 mg of dry gel) were dispensed into 0.7 × 4 cm polypropylene columns. Each column was then washed with 1 mL of 20 mM Tris-HCl buffer (pH 7.4) containing 10 mM theophylline and 1 mM mercaptoethanol (buffer A). One milliliter of the sonicated cell preparation was applied to each column, and the enzyme activity was recovered by stepwise elution with increasing concentrations of NaCl (0.1 to 0.5 M) in buffer A. For DEAE-cellulose chromatography, a 1 × 14 cm column of DEAE-cellulose was equilibrated with 10 mM Tris-HCl buffer (pH 7.4) at 7 °C. Two milliliters of the sonicated cell preparation (100 mg of protein), previously dialyzed against the same buffer, was applied to the column, which was washed with 20 mL of 10 mM Tris-HCl buffer (pH 7.4). The enzyme was then eluted with a gradient of NaCl or KCl from 0 to 500 mM, using a total volume of 100 mL. The use of a KCl gradient for ion-exchange chromatography of enzymes partially purified on Sephadex G-200 was based on the observation that KCl caused less inactivation of the holoenzymes than similar concentrations of NaCl.

Gel Filtration and Density Gradient Centrifugation. Columns of Sephadex G-100 and G-200 (0.9 × 100 cm) were equilibrated with buffer A at 4 °C and employed for gel filtration of partially purified interstitial cell extracts. The columns were calibrated with Blue Dextran and ^3H_2O to define V_0 and V_t, and with the following standard proteins: 7S γ-globulin, bovine serum albumin, human chorionic gonadotropin, human luteinizing hormone, hCG α subunit, cytochrome *c*, phosphorylase *b*, chymotrypsinogen A, and human hemoglobin.

Density gradient centrifugation was performed in 5 to 20% (w/v) sucrose in a Beckman Model L2-65B ultracentrifuge, employing the SW-40 or SW-65 rotor. Gradients were prepared with an LKB gradient former Model 11300 employing a linear mixing profile and reservoirs containing 5% and 20% sucrose in buffer A. After passage through a small mixing chamber, the stream was split by a three-channel Buchler polystaltic pump and directed into cellulose nitrate centrifuge tubes. Sucrose gradients were usually stored at 4 °C for 3 to 4 h before use, but could be used immediately if necessary. Sample solutions of up to 0.5 mL were applied after addition of protein markers, usually bovine serum albumin, 7S human γ-globulin and [^{125}I]hCG. After centrifugation, fractions were collected for timed intervals by aspiration through a glass capillary connected to a Buchler polystaltic pump. Protein was measured by the method of Lowry et al. (1951) and labeled peaks were located by determining the radioactivity present in each fraction, after addition of aliquots to xylene-based scintillation fluid (Aquasol, New England Nuclear), in a liquid scintillation spectrometer. In all fractionation procedures, protein kinase activity was measured immediately after eluent fractions were collected into 10% sucrose and 0.05% BSA to preserve enzymatic activity.

Binding Studies with ^{3}H-Labeled cAMP. cAMP binding studies were performed by incubation of 200 μL (240 μg of protein) aliquots of partially purified protein kinase (eluted from DEAE-Sephadex) with 200 μL of 10^{-10} to 10^{-12} M [^{3}H]cAMP in 50 mM phosphate buffer (pH 6.5) containing

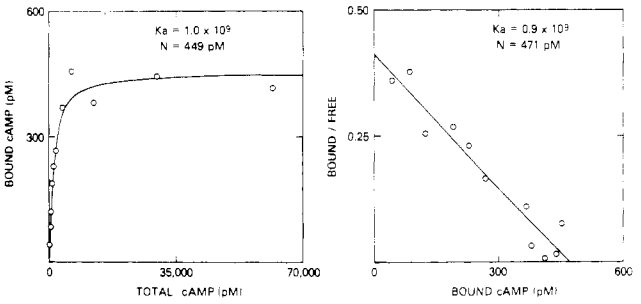

FIGURE 1: Saturation curve (left) and Scatchard plot (right) of cAMP binding data derived by equilibration of increasing concentrations of [^{3}H]cAMP with protein kinase receptor eluted from DEAE-Sephadex with 0.2–0.3 M NaCl.

10 mM theophylline, 10 mM magnesium acetate, and 10 mM mercaptoethanol, and 100 μL of buffer containing 10^{-3} M 1-methyl-3-isobutylxanthine. After incubation at 4 °C for periods up to 24 h, cAMP bound to protein kinase was isolated by adsorption to cellulose filters (Tao et al., 1970). After addition of 2 mL of ice-cold buffer, the contents of each tube were transferred to cellulose filters. The tubes were then washed twice with 2-mL aliquots of cold buffer, which were transferred to the respective filters. Each filter was washed twice with 2 mL of cold buffer, dried under suction, and dissolved in Aquasol prior to measurement of bound radioactivity in a liquid scintillation spectrometer (Beckman Model LS-250) with tritium counting efficiency of 50%. The mean nonspecific binding measured in the presence of 10^{-7} M cAMP was 2 fmol per incubation tube, and no change was observed with increasing amounts of [^{3}H]cAMP.

In certain experiments, interstitial cell sonicates were dialyzed against buffer A in washed cellophane tubing (Visking) for 16 h at 4 °C, in a total volume of 2 mL of buffer A containing 2.5 μCi of [^{3}H]cAMP and samples of partially purified protein kinase. After incubation of samples with the labeled nucleotide, aliquots were subjected to a variety of separation procedures including DEAE-Sephadex chromatography, gel filtration, sucrose gradient centrifugation, and Millipore filtration.

Calculation of Equilibrium and Kinetic Binding Constants. Equilibrium binding data were analyzed by the Scatchard method (Scatchard, 1949) or by an equation relating the concentration of bound ligand to the total cAMP concentration (Ketelslegers et al., 1975). Kinetic studies were analyzed with a nonlinear curve-fitting computer program, based upon the differential equation for the kinetics of a second-order chemical reaction (Ketelslegers et al., 1975). An interactive computer program with differential equation solving ability (Knott & Reece, 1972; Knott & Schrager, 1972) was used to perform all curve-fitting and calculations. Such programs were executed on a PDP-10 time sharing computer with graphic output facilities, via a Tektronix terminal 4010-1.

Results

Binding Properties of Partially Purified Protein Kinase. The rate of binding of [^{3}H]cAMP to protein kinase, partially purified by elution with 0.2–0.4 M NaCl from DEAE-Sephadex, was studied for various times up to 24 h at 4 °C in the presence of 0.1 mM methylisobutylxanthine. Maximum binding was observed after 5-h incubation, with no further change up to 14 h. The second-order association rate constant of the cAMP binding reaction at 4 °C was 2.1×10^6 M^{-1} min^{-1}. The first-order dissociation rate constant for release of cAMP from the regulatory subunit was 2.5×10^{-3} min^{-1}. The equilibrium constant calculated from the association and dissociation rate constants determined at 4 °C was 0.84×10^9 M^{-1}.

Equilibrium binding studies with [^{3}H]cAMP and partially purified protein kinase, eluted by 0.2–0.3 M NaCl from DEAE Sephadex, revealed a major set of binding sites with equilibrium association constant (K_a) of 1.1 ± 0.23 (SD) $\times 10^9$ M^{-1} (n = 6) (Figure 1). In addition to the predominant set of sites with K_a of about 10^9 M^{-1}, additional higher affinity sites with K_a of 1.3×10^{10} M^{-1} were sometimes detected in Leydig cell extracts. When present, such sites comprised only a small proportion (about 6%) of the total cAMP receptor concentration.

During binding–inhibition studies with the 0.2–0.3 M NaCl fractions from DEAE-Sephadex, the concentration of cAMP required for 50% inhibition of [^{3}H]cAMP binding was 0.3 nM. In the same experiment, competition with unlabeled cGmp gave a parallel binding–inhibition curve, with 50% binding at 3 μM cGMP. As these studies were performed under saturating conditions, the approximate binding affinity of the testis enzymes for cGMP was calculated from the binding–inhibition curves to be 10^5 M^{-1}.

Fractionation of Protein Kinase Holoenzymes: Binding of [^{3}H]cAMP and Phosphotransferase Activity. In previous studies based upon assays of the phosphotransferase activity of protein kinase, testicular interstitial cells were shown to contain two forms of cAMP-dependent holoenzyme with sedimentation coefficients of about 3.8 S and 6.5 S, respectively (Podesta et al., 1976b). The present experiments have identified and further characterized the two holoenzymes as 4.0S and 6.2S forms by cAMP binding studies and enzyme assays after Sephadex gel filtration, sucrose gradient centrifugation, and DEAE-cellulose chromatography.

When the 0.2 M and 0.3 M NaCl eluates from DEAE-Sephadex were analyzed separately by density gradient centrifugation, two discrete peaks of protein kinase activity were demonstrated. Centrifugation of the 0.2 M fraction on 5 to 20% sucrose for 20 h at 40 000 rpm in the SW-40 rotor showed a single 6.2S peak of cAMP binding activity. When the fraction eluted from DEAE-Sephadex by 0.3 M NaCl was centrifuged under the same conditions, assays of [^{3}H]cAMP binding and protein kinase activity in the eluted fractions showed single and coincident peaks of activity with sedimentation constant of 4.0 S. In the presence of saturating concentrations of unlabeled cAMP (10^{-7} M), the peak of [^{3}H]cAMP binding activity was completely abolished. The fraction eluted from DEAE-Sephadex with 0.3 M NaCl sometimes contained a small amount of the 6.2S holoenzyme. In such cases, [^{3}H]cAMP binding and protein kinase activity after sucrose density gradient centrifugation showed two regions of activity, a major peak corresponding to the 4.0S holoenzyme, and a minor 6.2S peak due to the presence of a small amount of the larger holoenzyme.

The hydrodynamic radii of the protein kinase holoenzymes were determined after analysis of purified Leydig cells extract by gel filtration on Sephadex G-200 (Figure 2, upper panel). This procedure revealed two main peaks of cAMP-dependent protein kinase activity which were eluted with partition coefficients (K_{av}) of 0.27 and 0.43. In each peak, enzymatic activity was coincident with the cAMP binding activity.

When extracts of purified Leydig cells were incubated with a saturating concentration of [^{3}H]cAMP (80 μM) for 16 h at 4 °C and further analyzed by gel filtration on Sephadex G-200 (Figure 2, lower panel), a single peak of cAMP-independent protein kinase activity was observed. The elution properties of this peak, with K_{av} of 0.56, corresponded to the free catalytic subunit formed after activation of the holoenzymes by unla-

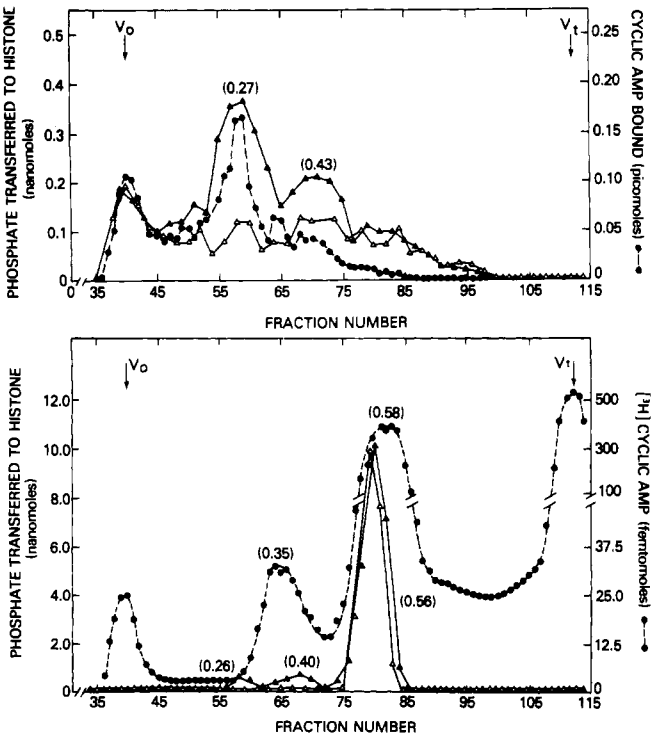

FIGURE 2: (Upper Panel) Gel filtration of cAMP binding (●) and protein kinase activities (△, − cAMP; ▲, + cAMP) in Leydig cell extracts analyzed by gel filtration on Sephadex G-200. After chromatography of the sonicated cell preparation, the eluent fractions were collected in 10% sucrose. For binding studies, aliquots were incubated with 1 nM (20 000 cpm) [^{3}H]cAMP in the presence and absence of 100 nM cAMP. Specific binding of [^{3}H]cAMP is shown in picomoles and the phosphotransferase activity in nanomoles; the K_{av} of each peak is indicated in parentheses. (Lower Panel) Gel filtration on Sephadex G-200 of regulatory and catalytic subunits of purified Leydig cell protein kinase. The sonicated cell preparation was incubated for 6 h with 80 μM [^{3}H]cAMP and then applied to the column. After fractionation, aliquots were analyzed for protein kinase activity in the presence or absence of cAMP. Other aliquots were counted to determine the [^{3}H]cAMP bound to regulatory subunits. Specific binding of [^{3}H]cAMP is shown as femtomoles and phosphotransferase activity is expressed in nanomoles; K_{av} of each peak is indicated in parentheses.

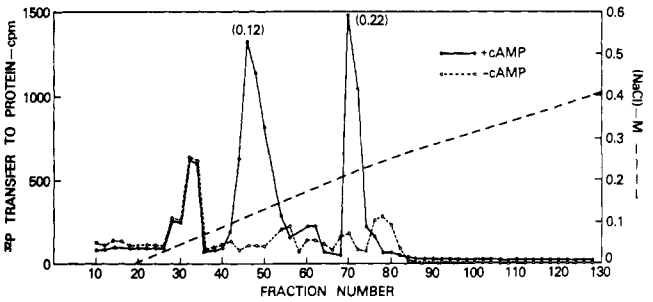

FIGURE 3: Ion-exchange chromatography of interstitial cell protein kinase, performed on a 1 × 14 cm column of DEAE-cellulose with elution by a linear gradient (0–500 mM) of sodium chloride.

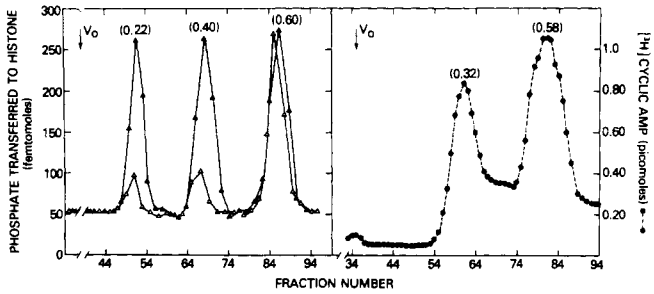

FIGURE 4: (Left Panel) Elution profile of protein kinase activity during gel filtration of the stored 6.2S holoenzyme on Sephadex G-200. Phosphotransferase activity was determined in the presence (▲—▲) or absence (△—△) of 10 μM cAMP. (Right Panel) Gel filtration of regulatory subunits formed during incubation of the 6.2S holoenzyme with a saturating concentration (80 μM) of [^{3}H]cAMP. Specific binding of [^{3}H]-cAMP is shown in picomoles (·- - -·).

beled cAMP. Two peaks of [^{3}H]cAMP binding were observed, with K_{av} values of 0.35 and 0.58, corresponding to the regulatory subunits of the holoenzymes. In addition, two very small peaks of residual cAMP-dependent protein kinase activity were eluted with K_{av} values of 0.26 and 0.40, similar to the activity profile observed during fractionation of the protein kinase holoenzymes (see Figure 2, upper panel).

Analysis of Leydig cell protein kinase by chromatography of extracts on DEAE-cellulose (Figure 3) revealed the presence of two cAMP-dependent peaks of enzyme activity. Upon fractionation of unstimulated Leydig cells, the holoenzyme peaks were eluted at NaCl concentrations of 120 mM and 220 mM, respectively. An additional small peak of cAMP-independent enzyme activity was eluted earlier from the DEAE-cellulose column at 50 mM NaCl, and corresponded to the free catalytic subunit formed by dissociation of the holoenzymes.

Because the second cAMP-dependent enzyme eluted from DEAE-cellulose and Sephadex gel was the smaller and less abundant of the two holoenzymes, experiments were performed to determine whether it was derived from the larger holoenzyme during preparation and fractionation of cell extracts. For this purpose, the 6.2S and 4.0S enzymes were resolved from Leydig cell extracts by gel filtration on Sephadex G-200, and the eluted fractions corresponding to the larger (K_{av} = 0.26) and smaller (K_{av} = 0.40) holoenzymes were pooled. Immediate rechromatography of the larger enzyme on Sephadex G-200 showed a major peak of enzyme activity with K_{av} of 0.26, and smaller peaks of enzyme activity with K_{av} values of 0.40 and 0.60. However, when an aliquot of the same fraction was kept for 16 h at 4 °C and subsequently analyzed on Sephadex G-200, the elution profile revealed three major peaks of enzyme activity. In addition to the original peak at K_{av} = 0.26, there was a further peak of cAMP-dependent enzyme activity at K_{av} = 0.40, and a peak of cAMP-independent protein kinase with K_{av} of 0.60 (Figure 4, left). To examine the corresponding regulatory subunit(s), the remainder of the pooled larger holoenzyme (K_{av} = 0.26) was incubated with a saturating concentration of [^{3}H]cAMP (80 μM) for 16 h at 4 °C to ensure dissociation of the holoenzyme. Subsequent fractionation on Sephadex G-200 revealed two peaks of [^{3}H]cAMP binding activity with K_{av} values of 0.32 and 0.58 (Figure 4, right). This elution pattern was similar to the behavior of the regulatory subunits upon filtration of the unfractionated Leydig cell extract on Sephadex G-200 (see Figure 2, lower panel).

Similar results were demonstrated upon fractionation of the individual holoenzymes by ion-exchange chromatography. In this case, during analysis on DEAE-cellulose of the 6.2S holoenzyme obtained by prior chromatography on Sephadex G-200, the three species of protein kinase were obtained (Figure 5, left). In addition to the main peak of cAMP-dependent protein kinase eluted by 110 mM KCl, two smaller peaks of activity were eluted to 50 mM and 210 mM KCl. The enzyme activity eluted at 50 mM KCl was not cAMP dependent and corresponded to the free catalytic subunit. The enzyme eluted at 210 mM KCl was cAMP dependent and represents the small holoenzyme that is presumably derived from

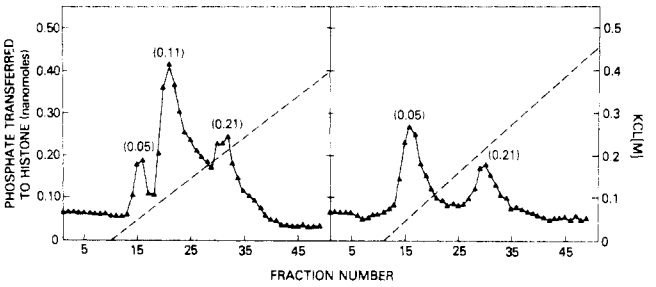

FIGURE 5: Ion-exchange chromatography of the larger (6.2S) holoenzyme (left panel) and the smaller (4.0S) holoenzyme (right panel) prepared by prior gel filtration of Leydig cell extracts on Sephadex G-200. Elution was performed with a linear gradient of 0–500 mM KCl.

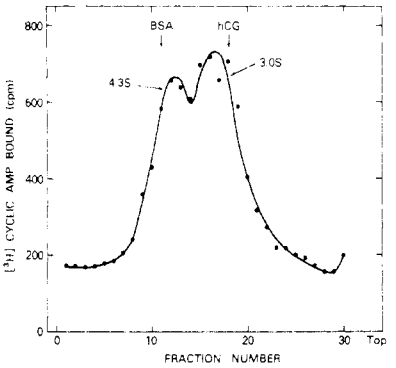

FIGURE 6: Sucrose density centrifugation of [^{3}H]cAMP bound to regulatory subunits of interstitial cell protein kinase. Centrifugation in 5–20% sucrose was performed for 20 h at 50 000 rpm in the SW-65 rotor.

the larger holoenzyme. In contrast, chromatography of the smaller holoenzyme (Figure 5, right) revealed only two peaks of activity, at 50 mM KCl and 210 mM KCl, corresponding to the free catalytic subunit and the small holoenzyme, respectively (see also Figure 3). These results indicate that the major cAMP-dependent protein kinase holoenzyme of the Leydig cell is the 6.2S form that is eluted by 110 mM KCl, similar to the type I enzymes described in other tissues. The presence of the smaller holoenzyme (4.0 S, eluted at 210 mM KCl) is probably the result of breakdown of the larger holoenzyme (see Figure 4). However, the presence of basal quantities of the small 4.0S holoenzyme in cell extracts, and of the small regulatory subunit in extracts incubated with cAMP prior to chromatography, could not be abolished when trypsin and chymotrypsin inhibitors, trasylol, and phenylmethanesulfonyl fluoride were present during disruption and Sephadex G-200 fractionation. Thus, the smaller enzyme could be derived by breakdown or dissociation of the 6.2S enzyme during in vitro analysis of Leydig cell extracts, but was also present in cells, presumably as an endogenous product of the larger holoenzyme.

Fractionation of Regulatory Subunits: Binding of [^{3}H]-cAMP. During initial studies performed to establish the elution profile of protein kinase regulatory subunits upon anion-exchange chromatography, protein kinase from sonicated Leydig cells was purified by stepwise elution from DEAE-Sephadex with increasing NaCl concentrations. Pools of the 0.2–0.3 M NaCl fractions were equilibrated with [^{3}H]cAMP by dialysis for 16 h at 4 °C, and again subjected to chromatography on DEAE-Sephadex. Analysis of bound radioactivity in the eluted fractions by cellulose filtration showed that the majority of the cAMP binding activity was recovered in the 0.3 M NaCl fraction. The regulatory subunits were also analyzed by gel filtration on Sephadex G-100, and by sucrose gradient centrifugation, followed by assay of receptor bound [^{3}H]cAMP in the individual fractions by cellulose filtration.

This fractionation procedure demonstrated the presence of two cAMP binding peaks, presumed to correspond to the regulatory subunits of the 6.2S and 4.0S holoenzymes, with K_{av} values of 0.17 for the larger subunit and 0.50 for the smaller subunit. The regulatory subunits were also demonstrated by sucrose density gradient centrifugation, performed for 20 h at 50 000 rpm in the SW-65 rotor, and followed by assay of cAMP binding activity in the individual fractions by cellulose filtration. The pattern of binding activity in the sucrose gradient was consistent with the presence of two regulatory subunits, with sedimentation constants of 4.2 S and 3.0 S (Figure 6). An identical sedimentation pattern, with 4.2S and 3.0S peaks of cAMP binding activity, was observed in extracts of isolated interstitial cells after exposure to a concentration of hCG (10^{-10} M) that produced maximum activation of protein kinase in vitro. Analysis of the two regulatory subunits on DEAE-cellulose revealed the presence of two peaks of cAMP binding activity eluted at KCl concentrations of 135 mM and 300 mM for the 4.2S and 3.0S regulatory subunits, respectively. In addition, when chromatography on DEAE-cellulose was performed after 16 h incubation prior to fractionation, only one peak of cAMP binding activity was eluted, at a KCl concentration of 300 mM. This finding is consistent with the results obtained by fractionation of cell extracts on DEAE-Sephadex after equilibration with [^{3}H]cAMP, and probably represents the complete conversion of the larger holoenzyme and/or regulatory subunit to the smaller regulatory subunit.

TABLE I: Physical Properties of Leydig Cell Protein Kinase Holoenzymes and Regulatory Subunits.[a]

	Sedimentation coefficient (10^{-13} s)	Stokes radius (10^{-8} cm)	Mol wt ($\times 10^{-3}$)
Holoenzyme I	6.2 ± 0.1 (3)	47.7 ± 1.6 (9)	116 (112–120)
Holoenzyme II	4.0 ± 0.1 (4)	37.9 ± 1.0 (5)	59 (57–61)
Regulatory subunit I	4.2 ± 0.2 (4)	42.1 ± 1.5 (5)	66 (63–69)
Regulatory subunit II	3.0 ± 0.1 (4)	30.1 ± 1.9 (5)	35 (32–38)
Catalytic subunit	2.9 ± 0.1 (3)	29.0 ± 1.5 (4)	33 (30–35)

[a] Values are mean ± standard deviation or mean and 99% confidence limits.

Physical Properties of Protein Kinase Holoenzymes and Their Regulatory and Catalytic Subunits. The Stokes radii of the holoenzymes and regulatory subunits calculated from gel filtration studies were 48 Å for the 6.2S and 38 Å for the 4.0S holoenzymes, and 42 Å for the 4.2S and 30 Å for the 3.0S regulatory subunits. The approximate molecular weights of the two holoenzymes calculated by combining data from gel filtration and sucrose gradient centrifugation (Siegel & Monty, 1966), were 116 000 and 60 000 respectively (Table I). The estimated molecular weights of the corresponding regulatory subunits were 66 000 and 35 000 respectively. The Stokes radius of the 2.9S catalytic subunit was 29 Å and the approximate molecular weight was 33 000.

Characteristics of Protein Kinase Holoenzymes. In keeping with the cAMP-dependent nature of the Leydig cell enzymes, the phosphotransferase activities of both holoenzymes and their common catalytic subunit were completely abolished by incubation with the heat-stable inhibitor protein (Ashby & Walsh, 1973). During fractionation of the Leydig cell enzymes

on DEAE-cellulose at pH 7.4, the larger 6.2S holoenzyme was eluted by 120 mM NaCl, with a similar elution profile to the type I protein kinase present in rodent heart and liver (Corbin et al., 1975). The catalytic subunit was adsorbed by DEAE-cellulose from 10 mM Tris-HCl buffer pH 7.4 and was eluted by 50 mM NaCl. This difference from the behavior of the catalytic subunits of rat heart and liver, which passed unadsorbed through DEAE-cellulose columns at pH 6.8 (Corbin et al., 1975) is attributable to the slightly higher pH (7.4) employed in the present study. Likewise, the salt concentration necessary to elute the major holoenzyme (120 mM) was slightly higher than that required to elute the type I enzymes of rodent heart and liver (100 mM) due to the higher buffer pH used for chromatography of the testicular enzymes.

The similarity of the first-eluted holoenzyme to the type I enzyme of other tissues was indicated by its charge and sedimentation characteristics, and also by salt-sensitivity and cAMP binding properties of the major Leydig cell enzyme. Thus, when the NaCl concentration was increased progressively in phosphokinase assays of the 6.2S enzyme (prepared by gel filtration of Leydig cell extracts) partial inactivation of the enzyme occurred up to 0.3 M NaCl, while dissociation and activation of the residual enzyme were evident at 0.4 to 0.5 M NaCl. In this regard, the 6.2S holoenzyme behaved as the type I enzyme present in rat heart and liver, rabbit skeletal muscle, and several other mammalian tissues (Nimmo & Cohen, 1977).

An additional feature of type I protein kinase is the ability of MgATP to influence subunit recombination and binding of cAMP to the holoenzyme (Hofmann et al., 1975). These effects of MgATP appear to be characteristic of enzymes eluted from DEAE-cellulose by low salt concentrations, such as those of rabbit skeletal muscle and rat heart. The binding affinity of the testis enzyme for cAMP was also markedly decreased by MgATP, with a fall of about 50-fold in K_a as MgATP concentration was increased from 10^{-8} to 10^{-6} M. Thus, the properties of the larger and predominant holoenzyme of the Leydig cell were identical in several regards with those of the type I enzyme in other tissues.

The characteristics of the smaller and less abundant 4.0S holoenzyme eluted by 220 mM NaCl from DEAE-cellulose have been less extensively analyzed, but were in general more similar to those of the type I enzyme than those of the type II enzyme eluted by high salt concentrations during DEAE-cellulose chromatography of rat adipose tissue and brain (Corbin et al., 1975) and bovine heart extracts (Hofmann et al., 1975). Thus, the 4.0S enzyme was found to be partially inactivated by rising NaCl concentration, and appeared to share the sensitivity of the 6.2S enzyme to MgATP during cAMP binding studies performed on Leydig cell extracts. For these reasons, the 4.0S enzyme was more similar to the type I enzyme than the type II form, other than in its more acidic charge and the correspondingly higher salt concentration required for elution from DEAE-cellulose. These common features are consistent with the possible origin of the 4.0S enzyme from the 6.2S holoenzyme. The tighter binding of the 4.0S enzyme to DEAE-cellulose may also be a reflection of this origin, if the regulatory (R) subunit of the smaller enzyme represents a dissociated and more acidic subunit of the (R2) regulatory portion of the 6.2S holoenzyme.

Discussion

These studies have defined the physical properties and binding characteristics of two cAMP-dependent protein kinases of the purified Leydig cells. The 4.0S and 6.2S holoenzymes possess approximate molecular weights of 60 000 and 116 000 and exhibit relatively high binding affinity for cAMP, with equilibrium association constant of 1.1×10^9 M^{-1}. A small number of binding sites of higher affinity (10^{10} M^{-1}) was sometimes demonstrable in testis extracts and represented up to 6% of the total cAMP receptor concentration. These experiments have also demonstrated the presence of two regulatory subunits which bind cAMP, a 3.0S species that corresponds to the 4.0S holoenzyme, and 4.2S subunit that corresponds to the larger 6.2S holoenzyme. The apparent molecular weights of the regulatory subunits were 35 500 and 66 000, and the molecular weight of the common 2.9S catalytic subunit was 33 000. This value is similar to that of the purified catalytic subunits of bovine liver protein kinase, with Stokes radius of 27.3 to 30.8 Å and molecular weight of 39 000 to 42 000 (Sugden et al., 1976).

The presence of two protein kinase holoenzymes which exhibit sedimentation characteristics similar to those observed in the interstitial cell has also been described in skeletal muscle, where conversion to separate regulatory subunits and a single catalytic subunit was observed on exposure to cAMP (Reimann et al., 1971). Previous estimations of the association constant (K_a) of protein kinase in steroidogenic tissues for cAMP have been of the order of 10^8 M^{-1} (Menon, 1973; Gill & Walton, 1974). The testicular enzymes exhibit relatively high binding affinity for cAMP (1×10^9 M^{-1}) by comparison with values observed in other tissues. The association rate constant of 2×10^6 M^{-1} min^{-1} derived at 4 °C is comparable with the value of 4.4×10^7 M^{-1} min^{-1} observed at 25 °C in the adrenal cAMP receptor by Gill & Walton (1974), since the reaction is markedly temperature-dependent and proceeds about ten times faster at the higher temperature. The dissociation rate constant of 4.3×10^{-3} min^{-1} is also comparable to values observed for the cAMP receptor protein of other tissues. Observations by Sanborn et al. (1973) upon the protein kinase of uterine muscle showed that dissociation rate constants were also markedly influenced by temperature and pH, with considerable reduction at more acid pH and at lower temperatures. The latter observers also described the presence of sites with higher affinity than of the predominant binding species. However, the presence of a protein kinase fraction with extremely high binding affinity for cAMP (i.e., about 10^{10} M^{-1}) has not been observed in the majority of binding studies reported on protein kinase derived from various tissues. The cyclic nucleotide binding specificity of the testis enzyme was relatively high, and equivalent degrees of binding-inhibition were observed with cGMP only at concentrations 10^4 times higher than the corresponding concentrations of cAMP.

Fractionation of the protein kinase holoenzymes by sucrose gradient centrifugation, ion-exchange chromatography, and gel filtration revealed that 70% of the total cAMP-dependent protein kinase activity was contributed by the 6.2S holoenzyme. This predominant form behaved during chromatography on DEAE-cellulose as the type I cAMP-dependent protein kinase described in several other tissues (Rubin & Rosen, 1975). In addition to the property of elution from DEAE-cellulose by low salt concentrations (120 mM NaCl), the major protein kinase holoenzyme was a 6.2S molecule and showed partial inactivation and dissociation by high salt concentration. These features, and the marked effect of MgATP upon the binding affinity of the holoenzyme for cAMP, are characteristic of the type I protein kinase found in several mammalian tissues (Nimmo & Cohen, 1977). The presence of the type I enzyme has been previously noted in extracts of the whole rat

testis (Corbin et al., 1975), but has not been previously demonstrated in the Leydig cells, which constitute only a small proportion of the rodent testis. It is of interest that the type I enzyme also appears to be the predominant form of protein kinase in the corpus luteum of the cow ovary (Menon, 1973), whereas the type II enzyme has been reported to be the major form in the calf ovary (Talmadge et al., 1975) and the bovine adrenal gland (Shima et al., 1975). Thus, as in striated muscle, there is no consistent distribution of the two holoenzymes in steroidogenic tissues.

The second protein kinase holoenzyme detected in rat Leydig cells was less abundant than the type I enzyme and was eluted from DEAE-cellulose by higher salt concentrations in a manner similar to the type II enzyme that predominates in bovine heart (Hofmann et al., 1975) and rat adipose tissue (Corbin et al., 1975). However, the minor Leydig cell enzyme was smaller than the type II enzymes (4.0S vs. 7.0S), and was similar to the type I enzyme in terms of salt sensitivity and regulation of cAMP binding by MgATP. These features, and the demonstration that the 4.0S enzyme was formed in vitro from the major 6.2S holoenzyme, indicate that the smaller enzyme present in Leydig cell extracts may be derived from the larger type I enzyme during cell fractionation. However, the 4.0S protein kinase was consistently present in Leydig cell extracts and represented about 30% of the total cAMP-dependent protein kinase activity. The presence of the 4.0S enzyme in cell extracts prepared in the presence of protease inhibitors during the fractionation procedure indicates that the smaller enzyme is probably a normal component of the protein kinase activity in Leydig cells.

The finding that the partially purified preparation of the larger holoenzyme could yield the three forms of protein kinase (i.e., the 6.2 and 4.0 S holoenzymes and the free catalytic subunit) suggests that the larger (6.2S) form exists as an R_2C_2 complex, and that the smaller (4.0S) is an RC form of protein kinase. This structural arrangement is also supported by the molecular weight estimates, which show the 6.2S enzyme to be about twice the size of the 4.0S enzyme. A similar model for trypsin effect on bovine liver protein kinase has been proposed by Sugden & Corbin (1976). The presence of a smaller form of protein kinase that is presumably derived from a large holoenzyme has also been described in the calf ovary, where a proteolytic conversion of the larger to the smaller enzyme has been proposed (Talmadge et al., 1977).

If the 6.2S and 4.0S protein kinase holoenzymes in the Leydig cell are equally and readily accessible to receptor-generated cAMP, then both enzymes would be activated simultaneously during hormone stimulation. However, preliminary studies in Leydig cell homogenates have shown that the 4.0S enzyme is confined to the membrane-rich cell fraction obtained by density gradient centrifugation, while both 4.0S and 6.2S holoenzymes are present in the cytosol (Podesta et al., unpublished observations). Alternatively, one of the enzymes could be located at a site which favors selective activation by minute changes or translocation of cAMP. We have previously demonstrated that simulation of total protein kinase activity was induced in parallel with increases in cyclic AMP by hCG concentrations which are supramaximal for testosterone production, and that 10^{-10} M hCG produced complete activation of both holoenzymes (Podesta et al., 1976b). It should now be possible to determine whether lower hCG concentrations, which stimulate steroidogenesis through very small increases in cAMP formation (Dufau et al., 1977), could selectively stimulate one of the protein kinase holoenzymes. If preferential activation of one form of protein kinase was evoked by low receptor occupancy, it is likely that the corresponding enzyme would be localized in a compartment in relation to cholesterol transport or key steroidogenic enzymes.

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Kinetic Studies on the Reactions Catalyzed by Chorismate Mutase–Prephenate Dehydrogenase from *Aerobacter aerogenes*†

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ABSTRACT: Steady-state kinetic techniques have been used to investigate each of the reactions catalyzed by the bifunctional enzyme, chorismate mutase-prephenate dehydrogenase, from *Aerobacter aerogenes*. The results of steady-state velocity studies in the absence of products, as well as product and dead-end inhibition studies, suggest that the prephenate dehydrogenase reaction conforms to a rapid equilibrium random mechanism which involves the formation of two dead-end complexes, viz., enzyme-NADH-prephenate and enzyme-NAD^+-hydroxyphenylpyruvate. Chorismate functions as an activator of the dehydrogenase while both prephenate and hydroxyphenylpyruvate acted as competitive inhibitors in the mutase reaction. By contrast, both NAD^+ and NADH function as activators of the mutase. Values of the kinetic parameters associated with the mutase and dehydrogenase reactions have been determined and the results discussed in terms of possible relationships between the catalytic sites for the two reactions. The data appear to be consistent with the enzyme having either a single site at which both reactions occur or two separate sites which possess similar kinetic properties.

Chorismate mutase-prephenate dehydrogenase from *Aerobacter aerogenes* (Koch et al., 1970a) is a bifunctional enzyme which catalyzes the reactions:

$$\text{chorismate} \xrightarrow[(1)]{} \text{prephenate}$$

$$\xrightarrow[(2)]{NAD^+} \text{4-hydroxyphenylpyruvate} + NADH + CO_2$$

Reaction 1 is catalyzed by chorismate mutase and reaction 2 by prephenate dehydrogenase. Both reactions are essentially irreversible. The enzyme has been reported to contain two very similar or identical subunits and to have a molecular weight of approximately 76 000 (Koch et al., 1970a,b).

Up to the present time, no clear indications have emerged as to what, if any, advantages have been conferred on microorganisms as a result of their possessing bifunctional enzymes which are composed of a single type of polypeptide chain and which catalyze sequential reactions (cf. Kirschner & Bisswanger, 1976). Further, studies on this type of enzyme have not been sufficiently extensive to determine if such bifunctional enzymes possess one or two catalytic sites and if channeling occurs between two separate sites. Attempts have been made to elucidate the relationship between the sites responsible for the mutase and dehydrogenase activities of chorismate mutase-prephenate dehydrogenase from *A. aerogenes* and *Escherichia coli* (Koch et al., 1972) but the findings were not definitive.

It appeared that kinetic investigations to determine the mechanism of and parameters associated with each reaction catalyzed by the enzyme from *A. aerogenes* might yield information relevant to these problems. Thus, it seemed that an answer to the question of the number of active sites might come from a comparison of the magnitudes of those kinetic constants which could be determined from kinetic studies on both the mutase and dehydrogenase reactions. Toward this end, investigations have been made of the inhibition of the mutase by prephenate and hydroxyphenylpyruvate as well as its activation by NAD^+ and NADH.[1] In addition, the kinetic mechanism of the dehydrogenase reaction has been determined and shown to be of the rapid equilibrium random type. From a comparison of the dissociation constants for the combinations of prephenate and hydroxyphenylpyruvate with free enzyme, as determined from investigations on the mutase and dehydrogenase reactions, it is concluded that the enzyne possesses either a single site or two separate sites with kinetic similarities.

Experimental Procedure

Materials

Protamine sulfate, *p*-phenylmethylsulfonyl fluoride, and dithiothreitol were obtained from Calbiochem. Hydroxylapatite was from Bio-Rad and cyanogen bromide activated Sepharose 4B from Pharmacia. Tris(hydroxymethyl)aminomethane (Tris) from Sigma was used during the enzyme purification but that used for kinetic studies was from Merck. AMP, NAD^+, and NADH were supplied by P-L Biochemicals, and 4-hydroxyphenylpyruvic acid, crystallized and lyophilized bovine serum albumin, and *cis*-aconitic acid by Sigma. All other commercial reagents were of the highest quality available.

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, (ethylenedinitrilo)tetraacetic acid.

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