Characterization of Leydig cell protein kinase

Further studies in hormone action

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Leydig cell cAMP-dependent protein kinase has been characterized using rapid fractionation and optimal conditions to minimize proteolysis. DEAE-cellulose analysis showed a single Type I peak of cAMP binding and enzyme activity that eluted at 0.1 M KCl. Photoaffinity labelling with 8-azido[^{32}P]cAMP followed by SDS–PAGE showed a doublet with M_r 54000 and 51000 for the peak fraction, while the original extract exhibited only the smaller form. Autophosphorylation revealed a doublet of M_r 54000 ± 573 and M_r 51000 ± 710. To titrate the occupancy of regulatory subunits during hCG action, free cAMP receptors were measured by 8-azido[^{3}H]cAMP binding under non-exchange conditions followed by photolysis. hCG treatment caused a dose-related decrease of free receptors and SDS–PAGE analysis of the 8-azido[^{32}P]cAMP regulatory subunit from control and hCG treated cells also showed a hormone dependent decrease in a single band of M_r 50000. These results have shown that the Leydig cell protein kinase behaves as a Type I enzyme on DEAE analysis, but has the physical characteristics of the Type II enzyme. The dose-dependent fall in available receptor sites during hCG stimulation further indicates the central role of cAMP in hormone action in the Leydig cell.

cAMP-dependent protein kinase *Leydig cell* *Hormone action*

1. INTRODUCTION

Leydig cells of the rat testis have been shown to contain two forms of cAMP-dependent protein kinase on analysis by DEAE-cellulose chromatography. The predominant Type I cytosolic protein kinase (as judged by chromatographic behavior) was eluted from DEAE-cellulose at 0.12 M KCl, and an apparently Type II eluting at 0.22 M KCl was regarded as a derivative of the Type I. The low M_r of the regulatory subunits under nondenaturing conditions (66000 for RI and 35000 for RII) indicated the presence of proteolytic degradation [1]. Since the earlier studies, techniques for large scale preparations of pure Leydig cells have been developed and here we have further characterized the Leydig cell enzyme under conditions that minimize proteolysis.

2. MATERIALS AND METHODS

Sprague-Dawley rats of 300 g were purchased from Charles River, Wilmington, DE. hCG (CR 121), 12000 U/mg (Center for Population Research, NIH, NICHD, Bethesda, MD), [γ-^{32}P]ATP, 20–50 Ci/mmol, [^{3}H]cAMP was obtained from New England Nuclear, Boston, MA, 8-azido[^{32}P]cAMP from ICN, Irvine, CA, and ATP, cyclic AMP, benzamidine, and EDTA from Sigma, St. Louis, MO. De-52 diethylaminoethyl (DEAE) cellulose was purchased from Whatman, Clifton, NJ, collagenase Type II and histone F2b from Worthington, Freehold, NJ. Bovine serum albumin (BSA) was from Armour Pharmaceutical, Kankakee, IL. Electrophoretic supplies were from Biorad, Richmond, CA, Nonidet P 40 from Shell, Bethesda, MD, ampholines (Servalytes) from Accurate Chemical Co., Hicksville, NY and urea,

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ultrapure, Schwarz Mann, Spring Valley, NY. The 0.45 μm filters were from Millipore, Bedford, MA.

Interstitial cells were prepared from rat testes by the collagenase dispersion as in [2] followed by purification of Leydig cells by metrizamide gradients [3]. The cells were resuspended in either 199-BSA for cell incubations or in 10 mM Tris (pH 7.4), 10 mM theophylline and 1 mM mercaptoethanol (column buffer), for DEAE-fractionation.

Cells were sonicated for 30 s on ice and subsequently were centrifuged at 300000 × g for 60 min. The supernatant was applied onto a 1 × 14 cm DEAE-cellulose DE-52 column, equilibrated with column buffer. The sample was eluted using a 0–0.5 M KCl gradient and fractions of 1 ml were collected into tubes containing 100 μl 500 mM benzamidine, 50 mM EDTA, 50% sucrose. Fractions were tested for [^{3}H]cAMP binding and protein kinase activity. All chromatographic steps were carried out at 4°C.

Determinations of cAMP binding activity were performed using a modification of [4,5] and for protein kinase activity using a modification of [1,6].

Autophosphorylation was performed essentially as in [7,8] at 2°C for 10 min and the reaction was terminated with the addition of SDS–STOP solution (28% glycerol, 5.7% SDS and 14% mercaptoethanol for final concentration of 9.5% glycerol, 1.9% SDS and 4.8% mercaptoethanol) heating at 100°C for 3 min. Analysis was performed using 10% SDS–PAGE and autoradiography. Peak fractions were photoaffinity labelled using 5 μl of 8-azido[^{32}P]cAMP in the presence and absence of 1 mM cAMP [8,9]. Sample was allowed to exchange in the dark at 4°C for 40 min and then was subjected to UV light (max. = 254 λ) for 10 min and samples were prepared for SDS–PAGE [10] as indicated above. Cells were resuspended in 199-BSA and aliquoted 2 × 10^6 cells per vial and incubated at 34°C for 1 h with hCG or buffer control followed by centrifugation at 200 × g at 2°C for 10 min. Supernatants were prepared for testosterone and cAMP analysis [5]. For quantitation of cAMP free receptor sites, cell sonicates were incubated at 4°C for 1 h in the dark under non-exchange conditions with 10^{-5} M 8-azido[^{3}H]-cAMP in the presence or absence of cold cAMP. Samples were exposed to UV light on ice and the assay was then filtered on 0.45 μ Millipore filters and washed with ice-cold buffer A. Also, a parallel set of samples that were not exposed to UV light were transferred to an ice bath for 70 min and subsequently filtered. Because the specific activity of the 8-azido[^{32}P]cAMP given by the manufacturer was only an approximation, it was not possible to obtain an accurate quantitation of the free cAMP receptor sites with this tracer and for this purpose, we only used the 8-azido[^{3}H]cAMP compound cited above. However, it was feasible to monitor gonadotropin-induced disappearance of available receptor sites from Leydig cells treated with a maximal cAMP stimulatory dose of hCG using SDS–PAGE and autoradiography.

3. RESULTS

To analyze the cAMP binding regulatory subunit of protein kinase in rat Leydig cells, cytosols were subjected to autophosphorylation and 8-azido[^{32}P]cAMP labelling. The autoradiographs, developed at one day, of the 10% SDS–slab gels in which these samples were electrophoresed, are shown in fig.1, top. In lane 7, the 8-azido[^{32}P]cAMP-labelled cytosols showed a major band at M_r 51000 with some minor degradation products at M_r 45000–36000. These bands were reduced in the presence of 1 mM cold cAMP during the labelling procedure, lane 8. Autophosphorylation has been shown to be a property of the Type II protein kinase holoenzyme [7]. This is distinguished from cAMP-dependent phosphorylation because autophosphorylation occurs at low temperature, low ATP concentrations, and is inhibited by cAMP. Purified Leydig cells cytosols, in the presence and absence of 1 mM cAMP, were incubated with 5.0 μM, 0.5 μM and 0.05 μM [γ-^{32}P]ATP, at 2°C for 10 min (fig.1, top, lanes 3,4) and showed a doublet at M_r 54000 and M_r 51000 which was reduced when cold cAMP was present, suggesting an autophosphorylation event. At the lower ATP concentration, 0.05 μM bands appeared faint. Upon longer exposure, 2 and 15 days (fig.1, bottom) these were clearly defined and similarly reduced by cAMP. At the higher [γ-^{32}P]ATP concentration (5.0 μM) such a reduction did not occur, indicating that in addition in this case some cAMP-dependent phosphorylation had occurred (fig.1, top).

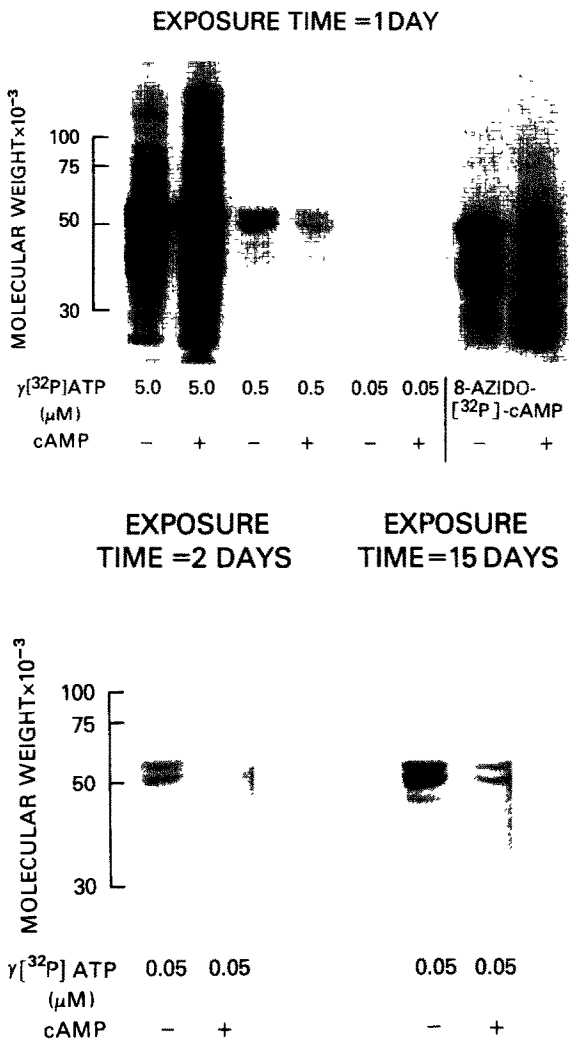

Fig.1. Top. Autoradiograph from SDS–PAGE of Leydig cell cytosols after (a) photoactivated incorporation of 8-N_3-[^{32}P]cyclic AMP into cyclic AMP binding protein; (b) autophosphorylation of cell extracts with 0.05–5.0 μM [^{32}P]ATP at 2°C for 10 min. Bottom. Autoradiograph of autophosphorylation experiment with 0.05 μM [γ-^{32}P]ATP as above, developed after 2 and 15 days.

Previous reports had demonstrated the presence of two holoenzymes in purified Leydig cells upon fractionation by ion exchange chromatography. The molecular masses of the Type II regulatory subunit were low [1,11] indicating proteolytic degradation of the enzyme subunit. DEAE-chromatography of the sample was examined without a prior prolonged dialysis step and in the presence of protease inhibitors. Using a 0–0.5 M KCl gradient, the holoenzyme eluted from the column in a single peak at 0.1 M KCl, as demonstrated by the specific [^{3}H]cAMP binding and cAMP-dependent protein kinase activity (fig.2). This would indicate that a Type I protein kinase (based on DEAE fractionation criteria) was present in purified Leydig cells. Also a peak of cAMP-independent protein kinase activity eluted at very low salt concentration and this is the catalytic subunit, since previous studies have shown a complete shift of the cAMP-dependent holoenzyme peak to a low salt peak of cyclic AMP independent activity upon fractionation of cytosols prepared from hormone stimulated Leydig cells [1,11].

In subsequent studies, the cAMP-dependent protein kinase activity peak fraction from the ion-exchange column was 8-azido[^{32}P]cAMP-labelled and analyzed on 10% slab-gel and compared with crude cytosols subjected to the photoaffinity labelling procedure. The autoradiograph (fig.3, lanes 1,2) shows a major band (M_r 51000) in the Leydig cell cytosols. In the eluted peak fraction from DEAE fractionation a doublet of M_r 54000 and M_r 51000 appeared labelled. The doublet was not observed when the peak fractions binding was performed in the presence of cold cAMP (fig.3, lanes 3,4). After an overnight dialysis step the 8-azido[^{32}P]cAMP-labelled sample was rechromatographed on an equilibrated DEAE-cellulose

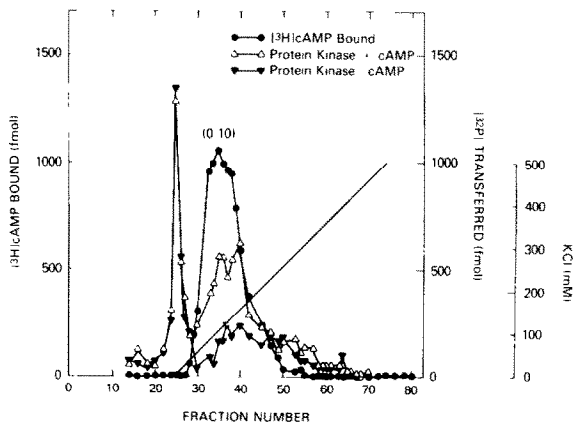

Fig.2. Ion-exchange chromatography column profile of cell extracts.

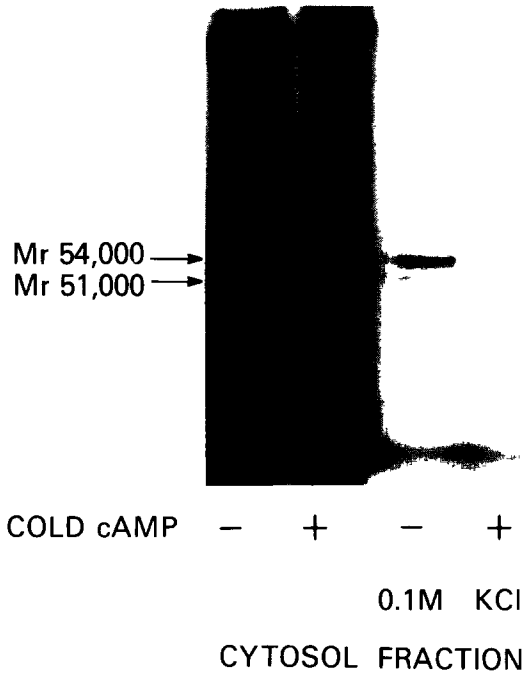

Fig.3. SDS–PAGE autoradiograph of photoaffinity labelled cytosol and of peak fractions from DEAE cellulose (0.10 M KCl) chromatography shown in fig.2. Aliquots from column fractions were subjected to autophosphorylation with [γ-^{32}P]ATP and photoaffinity labelling, followed by SDS–PAGE.

column and eluted with a 0–0.4 M KCl gradient. The column profile (fig.4) shows a major radioactive peak at 0.15 M KCl. When individual fractions were analyzed by SDS–PAGE, the cAMP-labelled regulatory subunit bands were apparent only in fractions in the region of the main radioactive peak at 0.15 M KCl. In this case only, a faint labelled band with M_r 51000 was observed, while the majority of labelled subunit was degraded and displayed M_r values of 41000 and 36000 (fig.5). As in previous experiments, the unfractionated cytosol showed a major band of M_r 51000 and two other bands of M_r 41000 and 36000.

The covalent linkage of the photoaffinity labelled probe provides an advantage over other ligands in binding assays. This technique was applied to titer the free cAMP-binding receptor sites in hormone-stimulated Leydig cells. Leydig cells were incubated with hCG and cell sonicates prepared as described in section 2 and cAMP binding assay with 8-azido[^{3}H]cAMP was performed

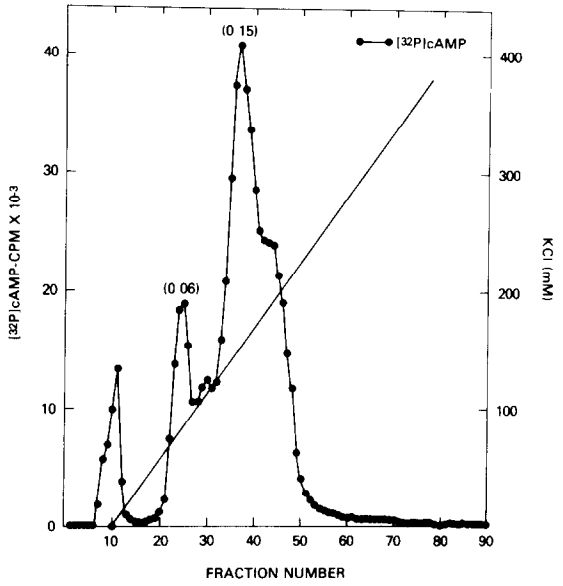

Fig.4. Column profile of 8-azido[^{32}P]cAMP-labelled regulatory subunit rechromatographed on DEAE-cellulose. Pool of aliquots from peak fractions of the holoenzyme resolved in DEAE chromatography was labelled and subjected to DEAE fractionation.

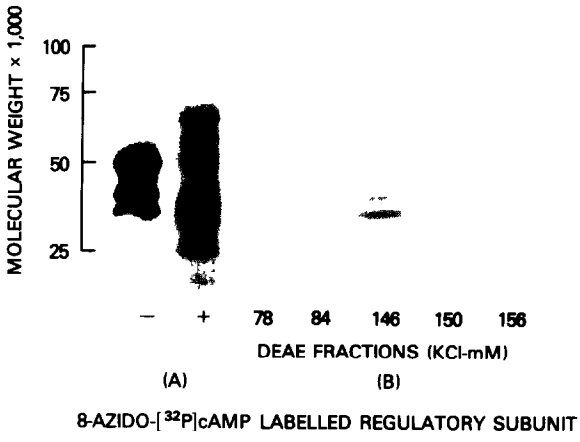

Fig.5. Autoradiograph of peak fractions of photoaffinity labelled regulatory subunit from column in fig.5. (A) Original non-fractionated cytosol. (B) DEAE column fractions.

under non-exchange conditions at 2°C (fig.6). Only half the samples were photolyzed and all the samples were filtered on 45 μm Millipore filters. The observed dose-dependent decrease in free cAMP receptors (from 0.64 ± 0.03 pmol/10^6 cells

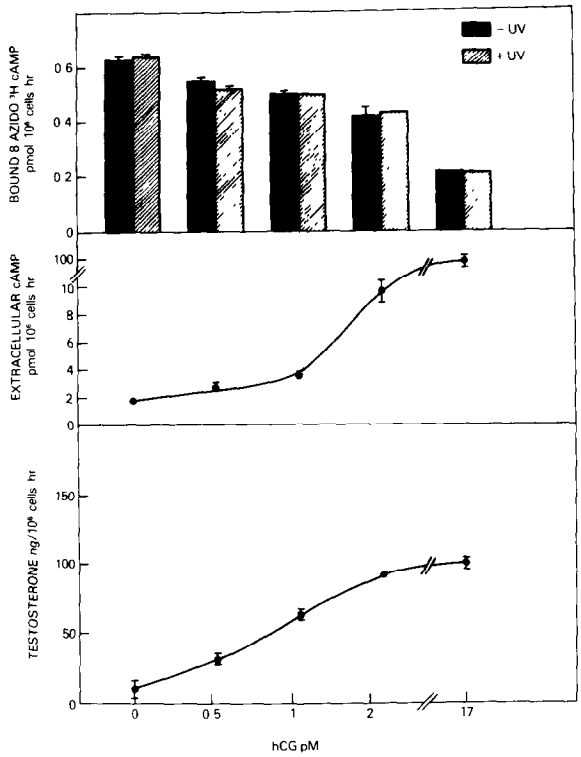

Fig.6. Determination of free cyclic AMP binding sites in Leydig cells incubated with hCG (0–17 pM). After incubation with or without hormone cells were subjected to binding assay (non-exchange conditions, 50 min at 40°C) with 8-azido[^{3}H]cAMP ± UV irradiation followed by Millipore filtration. Dose response of testosterone and cyclic AMP production of Leydig cells to hCG in vitro.

in control to 0.44 ± 0.03 pmol/10^6 cells at 2 pM hCG) was due to occupancy of receptors by endogenous cAMP produced by trophic hormone stimulus over the range of hCG concentrations. Extracellular cAMP production reached maximum at 17 pM hCG, where a reduction to 0.2 pmol/10^6 cells of free cAMP receptors was observed. Similar results were observed in UV exposed and unexposed samples. When control and 17 pM hCG treated samples aliquots were labelled with 8-azido[^{32}P]-cAMP, the autoradiographs showed a significant reduction of the labelled band in the sample of cytosol from cells treated with 17 pM hCG when compared to control level (lanes 1,3) and disappearance of the band when cold cAMP was included in the labelling procedure (fig.7, lanes 2,4). The above experiment provided a visual record of

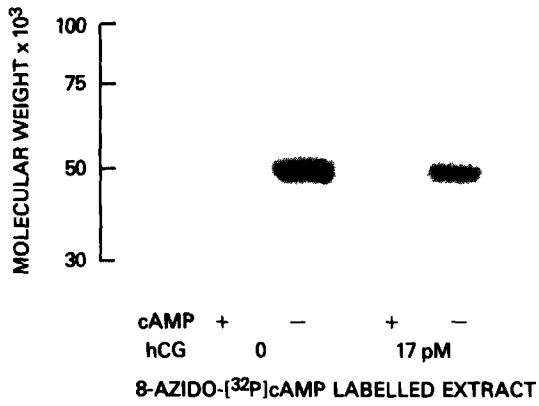

Fig.7. Autoradiograph of photoaffinity labelled regulatory subunit from control and hCG treated cells (17 pM).

the reduction in free receptor sites with increased occupancy of the receptor by endogenous cAMP during hormone action.

4. DISCUSSION

The analysis of the holoenzyme and regulatory subunit of protein kinase in purified Leydig cell has shown that these cells contain a Type I enzyme as shown by ion-exchange chromatography criteria but has the property of autophosphorylation, an attribute of Type II protein kinases. Autophosphorylation of the Leydig cell protein kinase, like the bovine myocardial protein kinase [6], occurred under the conditions of low temperature (2°C) and at low [γ-^{32}P]ATP concentrations (0.5 mM and 0.05 mM), and is inhibited by cAMP.

Previous studies in the rat Leydig cell protein kinase have demonstrated by DEAE analysis two regulatory subunits, Type I, eluting at 0.12 M NaCl with an M_r of 66300 and a Type II eluting at 0.22 M NaCl with M_r 35500 estimated under nondenaturing conditions. This suggested that the Type II enzyme was derived from the Type I, since the holoenzyme molecular mass did not correspond with those derived from other tissues [1,11]. This study has shown evidence to support this possibility, and has demonstrated an M_r for the regulatory subunit that is close to those previously reported in earlier studies for the type II enzyme [13]. By using highly purified Leydig cell prepara-

tions [3], reducing sample preparation time prior to chromatography, and eluting fractions into a protease inhibitor, benzamidine-EDTA, proteolysis has been minimized, hence the cytosolic fractions eluted from DEAE-cellulose under these conditions have only one peak of cAMP-dependent protein kinase activity at 0.1 M KCl.

Using purified bovine myocardial protein kinase, authors in [7] have observed two cAMP binding proteins using 8-azido[^{32}P]cAMP with M_r values of 52000 and 54000. Upon incorporation of a phosphate into the regulatory subunit of the 54-kDa band a shift to 56 kDa was described, while the 52-kDa band was unaffected [12]. In rat Leydig cell cytosol one major band at 51 kDa was observed with 8-azido[^{32}P]cAMP labelling. However, after ion-exchange chromatography, two bands were apparent with M_r values of 54000 and 51000. This would indicate that conversion of the 54-kDa band to 51 kDa occurred during the binding assay/photocrosslinking step in the crude cytosol. However, if rapid DEAE fractionation preceded the labelling of the receptor and this was followed promptly by SDS analysis the major labelled component was the 54-kDa species (fig.3). In the rat Leydig cell whether the M_r 54000 of this doublet is the result of phosphorylation is strongly suggested, since comparable bands were observed during autophosphorylation studies in unfractionated cytosols.

In previous studies, the progressive occupancy of the cAMP receptor protein after hCG stimulation of Leydig cells was quantitated using radioimmunoassay to measure the amount of bound cAMP, and [^{3}H]cAMP binding assay followed by Millipore filtration to measure free receptor sites in Leydig cell extracts [5]. The rat Leydig cell protein kinase is present only in very small quantities and is particularly unstable, degrading in 24–48 h. In light of these difficulties and the impossibility to obtain adequate quantities of pure undegraded material for characterization studies, we pursued further quantitation of free cAMP receptors using the photoaffinity probe. Binding assays performed as described in section 2 gave similar results for the number of free receptors when using 8-azido[^{3}H]-cAMP with or without photolysis. Furthermore, our results on the quantitation of free receptors during hormone action were similar to those obtained previously using [^{3}H]cAMP [5]. In all cases the absolute numbers of basal cAMP receptors and their subsequent reduction during occupancy by endogenous cyclic AMP during hormone action were identical.

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