ADENOSINE 3',5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE OF LEYDIG CELLS: IN VITRO ACTIVATION AND RELATIONSHIP TO GONADOTROPIN ACTION UPON CYCLIC AMP AND STEROIDOGENESIS

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1. Introduction

Gonadotropic stimulation of steroidogenesis in dispersed interstitial cells from the rat testis is elicited by subnanogram levels of LH or hCG, without a detectable change in cyclic AMP formation [1–4]. These observations have suggested that the steroidogenic action of gonadotropins may not operate via adenylate cyclase, or that steroidogenesis is activated by extremely small changes in cyclic AMP, possibly by translocation within a small intracellular pool. Since activation of protein kinase is the primary mechanism by which cyclic AMP controls the metabolic functions of eukaryotic cells, we have examined the in vitro actions of gonadotropin upon cyclic AMP-dependent protein kinase during homone-induced steroidogenesis in dispersed interstitial cells.

2. Materials and methods

Dispersed interstitial cells were prepared from testes of adult Sprague-Dawley rats by collagenase treatment as previously described [3,5–7]. Cell incubations were performed in triplicate at 34°C under 95% O_2:5% CO_2 in polyethylene vials containing about 40 million cells in 4 ml of Medium 199, 0.1 ml of hormone solution and 0.2 ml of 2 mM 1-methyl 3-isobutylxanthine. After incubation, 500 μl of the cell suspension was taken for measurement of cyclic AMP and testosterone production by radioimmunoassay as previously described [1]. The remainder of the cell suspension was centrifuged at

1200 rev/min for 10 min, and the cells were resuspended in 20 mM Tris–HCl buffer, pH 7.4, containing 1 mM 2-mercaptoethanol and 10 mM theophylline, then sonicated for 15 s.

For partial purification of protein kinase by ion-exchange chromatography, 30 mg aliquots of DEAE–Sephadex A50 in distilled water were dispensed into 0.7 × 4 cm polypropylene columns, then washed with 1 ml of Tris–HCl buffer. 1 ml of cell sonicate was applied to each column, washed with 1 ml of 20 mM Tris–HCl buffer solution, and the enzyme activity was eluted with 1 ml of 0.5 M NaCl in 20 mM Tris–HCl buffer, pH 7.4, containing 1 mM mercaptoethanol and 10 mM theophylline.

Assay of protein kinase was performed by a minor modification of the method of Reddit et al. [8], using histone F2b as a substrate in the presence or absence of 4 μM cyclic AMP.

3. Results

3.1. Properties of partially purified protein kinase

The partially purified protein kinase catalyzed the phosphorylation of casein, protamine and a number of histones, in the presence and absence of cyclic AMP. Significant activation of phosphorylation by cyclic AMP was observed when calf thymus histone F_{2b} or protamine were used as substrates. Protein kinase activity showed a pH optimum of 6.5 in the presence of 4 μM cyclic AMP. Enzyme activity was maximum at 100 mM NaCl, and was decreased by further increases in ionic strength. In the presence
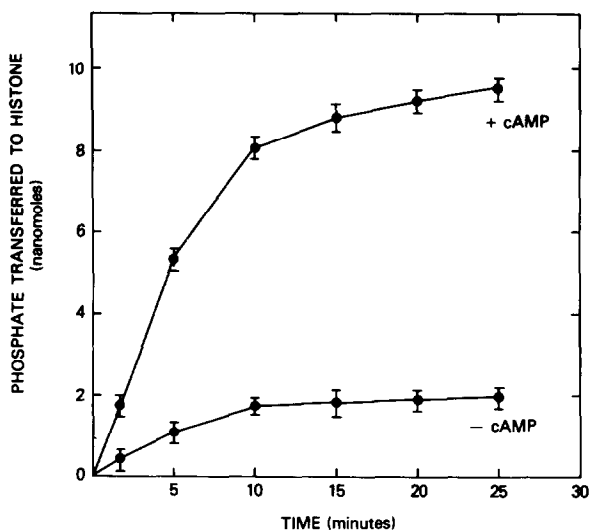
Fig.1. Time course of histone phosphorylation by protein kinase in the presence and absence of cyclic AMP. Each point represents the mean ± SD four determinations.
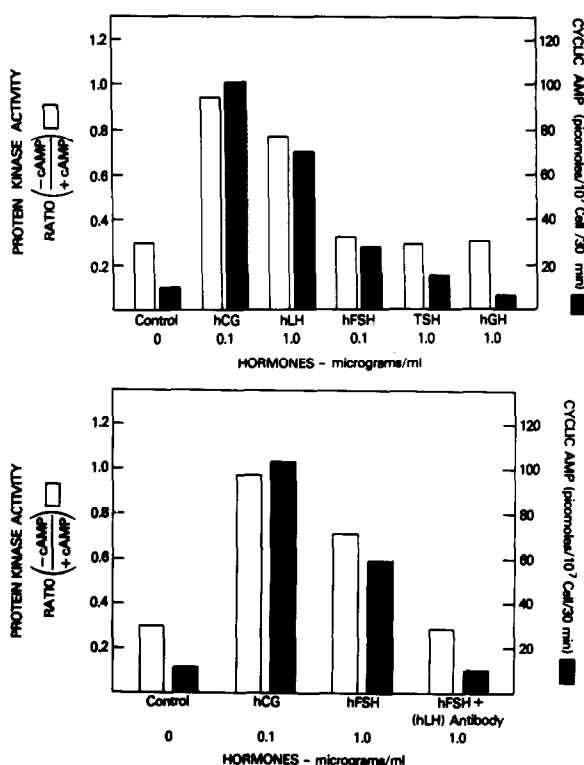

Fig.2. Cyclic AMP production and protein kinase activity during incubation of interstitial cells with pituitary hormones (upper panel). The effect of preincubation of hFSH preparations with antibody to hLH is shown in the lower panel.
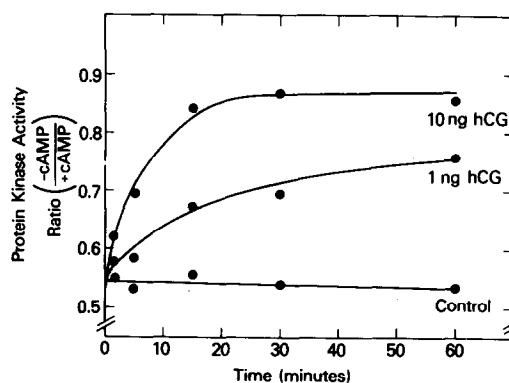
of 4 μM cyclic AMP, the enzyme reaction was linear for 5–10 min (fig.1). The approximate K_m for ATP in the presence or absence of cyclic AMP was 4×10^{-4} M; enzyme activity was maximum at 250 nM cyclic AMP, remained constant from 0.25–8 μM cyclic AMP, and decreased at higher concentration. Half-maximum activation of protein kinase was produced by 35 mM cyclic AMP in the presence of 1 mM ATP, with histone F2b as substrate.

3.2. Hormonal activation of protein kinase in vitro

Protein kinase activity was markedly increased when interstitial cells were incubated with 0.1 μg hCG or 1.0 μg hLH for 30 min. No stimulation of enzyme activity was observed in cells incubated with 0.1 μg hFSH, 1.0 μg hTSH and 1.0 μg hCG. Cyclic AMP production was significantly increased by hCG and hLH, and a minor increase of cyclic AMP production was observed with 0.1 μg hFSH and 1.0 μg hTSH (fig.2, upper panel). 1.0 μg hFSH caused a further increase of cyclic AMP, and evoked a significant increase in protein kinase activity. These effects of hFSH and hTSH were attributable to contamination with LH, being abolished by preincubation with specific antiserum to hLH (fig.2, lower panel). In the presence of 1 ng hCG/ml (10^{-11} M), in vitro activation

Fig.3. Stimulation of protein kinase activity during incubation of interstitial cells with 1 ng hCG/ml (10^{-11} M) and 10 ng hCG/ml (10^{-10} M) for time periods up to 60 min. Each point represents the mean of closely agreeing duplicates (± 5%).
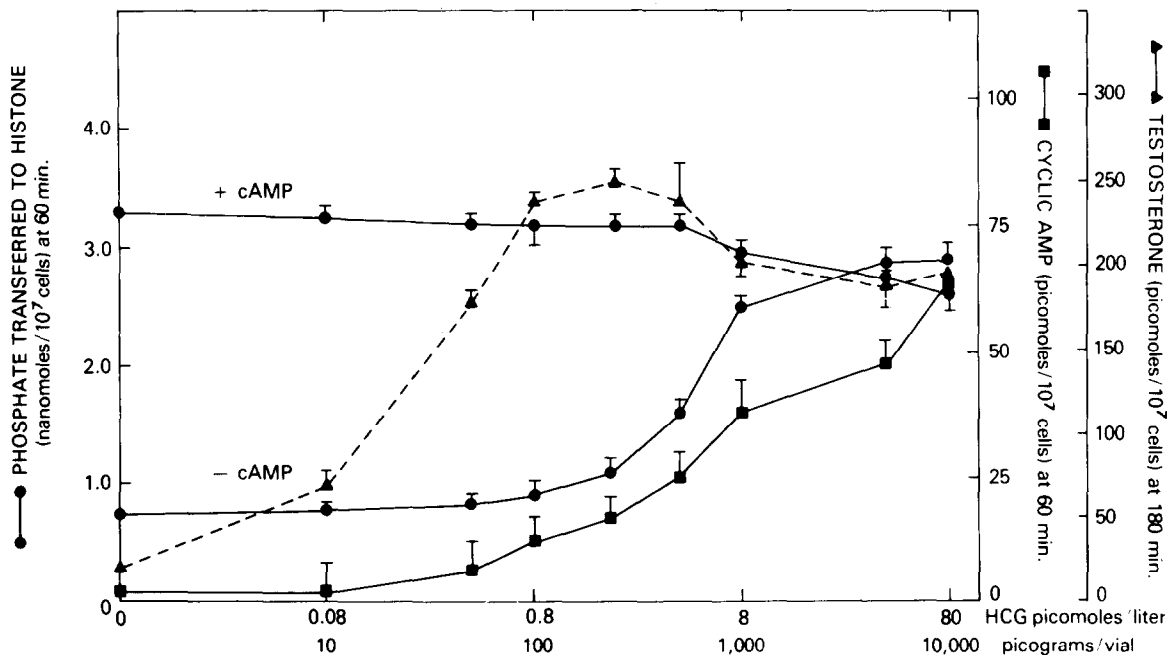
Fig.4. Cyclic AMP production, protein kinase activity and testosterone production during incubation of interstitial cells for 60 min with increasing concentrations of hCG. Each point represents the mean ± SD of quadruplicate incubations.

of protein kinase was apparent during incubation for 60 min at 35°C. A higher hCG concentration (10 ng/ ml, 10^{-10} M) caused enzyme activation within 2 min, with increase to a maximum at 15 min (fig.3). The intracellular concentration of cyclic AMP was also increased at 2 min, and reached a maximum after 15 min. Extracellular cyclic AMP levels were increased after 5 min, and rose substantially during incubation for 60 min. Leydig cells were also incubated with $0.1-100 \times 10^{-12}$ M hCG for 60 min, followed by measurement of cyclic AMP production, protein kinase activity and testosterone production. The dose—response curves for testosterone synthesis were from 5—100 pg hCG/ml (i.e. $10^{-13}-10^{-12}$ M), with half-maximum stimulation at 25 pg hCG ml. Cyclic AMP-dependent protein kinase of the Leydig cell was fully activated by incubation in vitro with 10 ng hCG/ml. The enzyme response to gonadotropin was detectable at 100—250 pg hCG and became maximum at 5 ng hCG/ml, with half-maximum stimulation at 1 ng/ml. This range of hCG concentration was identical with that required for stimulation of cyclic AMP production, demonstrating a close correlation

between hormonal induction of cyclic AMP synthesis and activation of protein kinase (fig.4).

3.3. Validation of hormonal activation of protein kinase

Additional experiments were performed to abolish or minimize the association or dissociation of protein kinase during cell sonication and extraction, activation of protein kinase in gonadotropin-treated cells was shown to be attributable to intracellular dissociation of the holoenzyme by endogenous cyclic AMP formed in response to gonadotropin. First, sonication was performed in the presence of 0.5 M NaCl to prevent reassociation of enzyme subunits which could have masked a minor degree of enzyme activation in response to increasing hCG concentration was unaltered when cell extracts were prepared in the presence of 0.5 M NaCl (table 1). Second, sonication of cells in the presence of a charcoal concentration (5 mg/ml) which bound 1000 pmol of cyclic AMP in one min. This procedure had no effect upon the activation of protein kinase observed with increasing gonadotropin concentrations (table 2).

Table 1

Gonadotropic stimulation of protein kinase in Leydig cells. Cell disruption was performed in the absence and presence of 0.5 M NaCl

Concentration of hCG (ng/ml)	Protein kinase activity ratio (−cAMP/+cAMP)	
	Without 0.5 M NaCl	With 0.5 M NaCl
0	0.23 ± 0.01	0.31 ± 0.04
0.01	0.24 ± 0.02	0.32 ± 0.02
0.02	0.22 ± 0.02	0.32 ± 0.03
0.10	0.25 ± 0.02	0.32 ± 0.02
1.00	0.72 ± 0.09	0.81 ± 0.07
10.0	0.77 ± 0.04	0.86 ± 0.06

Data is expressed as the mean ± SD of results from triplicate incubations, from which protein kinase assays were performed in duplicate.

These experiments served to validate the conclusion that intracellular activation of protein kinase was a consequence of cyclic AMP formation during stimulation of interstitial cells by activation observed during stimulation of dispersed Leydig cells by increasing hCG concentrations in vitro.

4. Discussion

These observations have demonstrated that protein kinase in isolated interstitial cells is activated in vitro by gonadotropin concentrations which are commensurate with those present in the circulation (10^{-11} M). Therefore, the protein kinase activity of testis interstitial cells in vivo is probably controlled by the plasma concentration of gonadotropic hormone. The high sensitivity of the phosphokinase activity of interstitial cell to gonadotropins illustrates that protein kinase activation is an important facet of the action of gonadotropin upon the Leydig cell.

In several hormone-sensitive tissues, activation of protein kinase has been shown to occur in parallel with the hormone-induced rise in cyclic AMP formation [9–11]. A similar correlation between cyclic AMP and enzyme activation was clearly apparent during the present studies with dispersed interstitial cells. However, there is evidence in the testis and other tissues for a dissociation between cyclic AMP production and subsequent hormonal responses. Such a disparity has been observed during studies on the effect of ACTH upon the adrenal [12], TSH upon the thyroid [13], and glucagon and epinephrine upon the liver [14], and is particularly marked in the interstitial cells of the testis [1–4]. In collagenase-dispersed rat interstitial cells, the maximum steroido-

Table 2

Gonadotropic stimulation of protein kinase in Leydig cells. Cell disruption was performed in the absence and presence of charcoal (5 mg/ml)

Concentration of hCG (ng/ml)	Protein kinase activity ratio (−cAMP/+cAMP)	
	Without charcoal	With charcoal
0	0.29 ± 0.01	0.29 ± 0.02
1	0.75 ± 0.04	0.78 ± 0.01
10	0.82 ± 0.08	0.78 ± 0.08
100	0.83 + 0.01	0.83 + 0.01

Data is expressed as the mean ± SD of results from triplicate incubations, from which protein kinase assays were performed in duplicate.

genic response is evoked by gonadotropin concen-
trations which do not produce a detectable change in
cyclic AMP formation [4,7]. Such interstitial cells,
like the other tissues mentioned above, show promi-
nent increases in cyclic AMP production when exposed
to higher hormone concentrations.

Even if the steroidogenic action of gonadotropin
is mediated by an 'undetectable' increment or turn-
over of cyclic AMP at low hormone concentrations,
the protein kinase activity of the target cell should
reflect such an effect of cyclic AMP. The present
experiments have confirmed the dissociation between
cyclic AMP and testosterone production, and have
shown that the dose—response curve for protein
kinase is obtained over the same hCG concentrations
which elicit a detectable increase in cyclic AMP
production. The relationship of protein kinase
activation to the measurable cyclic AMP levels
indicates that enzyme activation during gonadotropin
stimulation is directly related to the observed changes
in cyclic AMP concentration, and does not support
the existence of undetectable but functionally
important changes in cyclic AMP.

These findings suggest that stimulation of
steroidogenesis by low hormone concentrations
might act through a pathway independent of cyclic
AMP synthesis and activation of protein kinase.
However, it remains possible that translocation of
small quantities of cyclic AMP in a peri-membranal
functional compartment could activate a protein
kinase of high affinity and/or high substrate specificity.
For this reason, further studies on the phosphoryla-
tion of relevant substrates, such as enzymes and
transport proteins concerned in steroid synthesis,
will be necessary to clarify the role of cyclic AMP and
protein kinase in the regulation of steroidogenesis by
gonadotropic hormones.

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