GONADOTROPIN-INDUCED PHOSPHORYLATION OF ENDOGENOUS PROTEINS IN THE LEYDIG CELL

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1. Introduction

The actions of trophic hormones upon steroidogenic tissues are initiated by binding of the hormonal ligands to specific, high-affinity receptors in the target-cell plasma membrane. In the Leydig cell, the acute steroidogenic response to trophic hormone stimulation appears to be mediated by a highly compartmentalized activation pathway, in which minute increments in cAMP production cause a dose-related increase in protein kinase activity and testosterone biosynthesis [1–5]. These changes are preceded by corresponding increases in the binding of cyclic AMP to the regulatory subunit of the predominant type I protein kinase holoenzyme of the Leydig cell cytosol [1,6]. To further analyze the hormonal control of steroidogenic enzyme activity, we investigated the consequences of cyclic AMP-induced activation of protein kinase during gonadotropin action. This was performed by an initial analysis of the cyclic AMP-dependent phosphorylation of endogenous substrates in a cell-free system, followed by studies on LH/hCG or dibutyryl cyclic AMP-induced phosphorylation of proteins in isolated Leydig cells.

2. Materials and methods

2.1. *Preparation and incubation of Leydig cells*

Leydig cells were prepared from testes of 50-day old rats by dispersion with collagenase as in [7]. Cells were incubated with purified hCG (CR119, 10 000 IU/mg, or dibutyryl cyclic AMP (Bt_2cAMP; Becton Dickenson and Co, Rutherford NJ). Testosterone and cyclic AMP production were measured in incubation media by direct radioimmunoassay as in [1,7].

2.2. *Phosphorylation of endogenous substrates*

Leydig cells (9×10^8) were centrifuged at 120 g for 10 min and the cell pellet weighing 3.9 g was frozen and thawed 3-times in dry ice-acetone. The residue was taken up in 50% (w/v) 20 mM Tris–HCl buffer (pH 7.4) containing 10 mM theophylline and 1 mM mercaptoethanol (buffer A), homogenized in a Potter-Elvehjem glass tissue homogenizer, and centrifuged at 360 000 × *g* for 90 min. Aliquots of cytosol (100 μl) containing 300 μg protein, were incubated at 30°C with 5–10 μM [^{32}P]ATP (25–30 Ci/mmol, New England Nuclear, Boston MA) prepared in 100 μl buffer B (80 mM potassium phosphate, 10 mM magnesium acetate, 10 mM theophylline, 10 mM mercaptoethanol (pH 6.5)) in the presence or absence of 50 μM cyclic AMP. The phosphorylation reaction initiated by the addition of [^{32}P]ATP was performed for either 15–60 s (shorter time course) or 1–30 min (longer time course). The sample was then brought to a final concentration of 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol and 10% glycerol, and heated at 100°C for 15 min. Aliquots of 250 μl were then analyzed by electrophoresis on SDS–10% polyacrylamide slab gels [8]. For radioautography, dried gels were exposed to sheets (usually 4) of X-ray film (Kodak XR), which were then developed in a Kodak X-omat rapid developer. Estimates of the molecular mass (M_r) of phosphorylated proteins in SDS slab gels were obtained from plots of log M_r *vs* relative mobilities of standard proteins. Absorbance tracings of the autoradiograph were made using the relative absorbance at 633 nm with a Zeineh Soft Laser scanning microdensitometer (LKB, Instruments, Rockville MD).

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2.3. *Endogenous protein phosphorylation in intact cells*

Leydig cells were resuspended in 5 mM Hepes buffer (pH 7.4) containing 140 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.8 mM glucose and 0.1% bovine serum albumin, to give a final concentration of 10% (w/v). Aliquots of 2 ml containing 10^7 cells were incubated for 30 min with 1 mCi [^{32}P]-orthophosphoric acid (50 mCi/mmol, New England Nuclear, Boston MA) at 34°C. After this preincubation, cells were diluted 5-fold with Hepes buffer to 3.9×10^6 cell/ml and incubated in the presence or absence of hCG or Bt_2cAMP. The total incubation mixture volume of 220 μl, was composed of 200 μl ^{32}P-prelabeled cells, hCG or Bt_2cAMP in 10 μl Hepes buffer, and 10 μl 2.75 mM MIX. The incubation was terminated by addition of SDS stopping solution and subsequently analyzed by 10% SDS slab gel electrophoresis as in [8] followed by autoradiography. In other experiments, the reaction was stopped by addition of 100 μl Hepes buffer containing 9.5 M urea, 2% NP-40 (Nonidet P40, Shell, Bethesda Research Lab; Rockville MD) 2% ampholines of pH range 3–10 (LKB, Rockville MD) and 5% mercaptoethanol. Samples were kept at 0°C and analyzed by two-dimensional gel electrophoresis and autoradiography [9]. Isoelectric focusing was performed in 7.5% polyacrylamide gels containing 8 M urea, 2% pH 3–10 ampholytes, and 5% glycerol. The gels were overlayered with 6 M urea and polymerized for 2 h at room temperature, then samples were applied and overlayered with 6 M urea and 10% ampholines. The gels were run at 10°C for 16 h at 300 V. After electrofocusing, the gels were removed and the pH gradient was measured at 22°C using a surface electrode (Ingold Electronics) and a Beckman pH meter. Gels were then equilibrated with SDS sample buffer C (10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol, 2.3% (w/v) SDS in 62.5 mM Tris–HCl buffer (pH 6.8)) for 1 h at room temperature, and subjected to SDS gel electrophoresis and autoradiography.

3. Results

Initial studies were performed to characterize the endogenous substrates phosphorylated by cyclic AMP-dependent protein kinase in a cell-free system. Incubation of Leydig cell extracts with cyclic AMP for 15–60 s caused phosphorylation of 4 cyclic AMP-dependent bands of M_r 21 000, 25 000, 33 000 and 37 000 (fig.1, above) and two minor bands of M_r 66 000 and 105 000, with maximum phosphorylation at 30 s (fig.1, below). In addition, two cyclic AMP-independent phosphorylated bands were observed with M_r 12 000 and 16 000. During a longer time course of 1–30 min, cyclic AMP-stimulated phosphorylation of additional cytosol proteins of M_r 12 000, 15 000, 29 000 and 170 000 (not shown), corresponding to bands 10, 9, 6 and 1. Proteins of

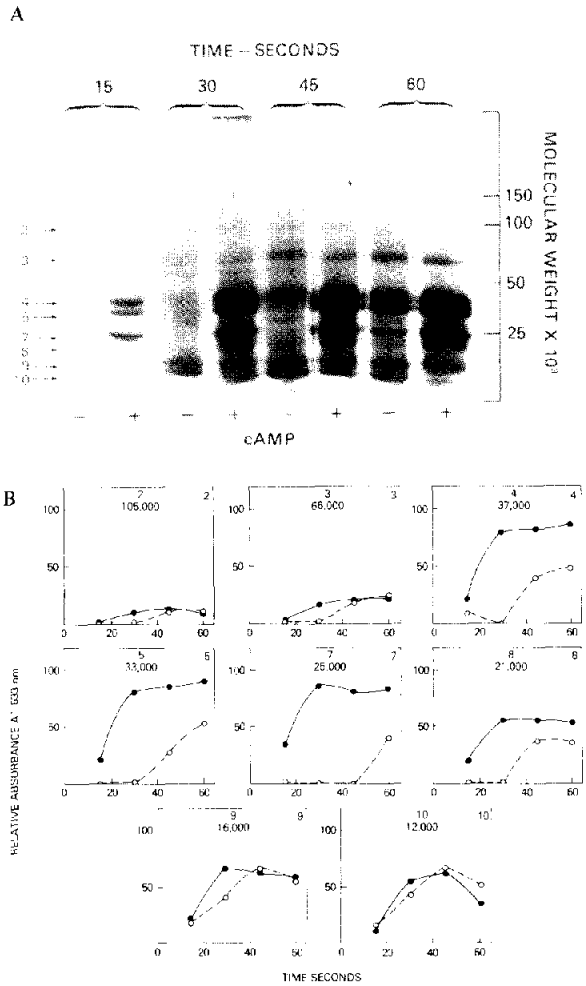

Fig.1. Cyclic AMP-induced phosphorylation of protein substrates in Leydig cell extracts. Above is shown an autoradiograph of SDS–polyacrylamide gel electrophoresis of Leydig cell extracts incubated with [^{32}P]ATP for 15–60 s in the presence (●——●) or absence (○– – –○) of 50 μM cAMP. The labeled proteins are numbered 1–10 on the left and these numbers are indicated on the corresponding plots of densitometric measurements of the gel autoradiograph shown below. Values were expressed as relative absorbance at 633 nm.

M_r 29 000 and 170 000 were not phosphorylated during the shorter time course; those of M_r 12 000 and 15 000 were phosphorylated at the earlier times but did not then appear to be cyclic AMP-dependent, and the corresponding densitometric measurements showed that phosphorylation of these proteins reached a maximum at 5 min.

During stimulation of Leydig cells with hCG (0.03–6 pM) for 10 min, a dose-related increase in phosphorylation was observed in 10 bands of M_r 22 000, 25 000, 30 000, 39 000, 42 000, 53 000, 58 000, 62 000, 75 000 and 90 000 (fig.2, above, numbered 10–1, respectively). The maximum hCG-stimulated phosphorylation was increased 0.5–6-fold over control levels, and the ED_{50} for hCG was 0.10–0.3 pM (fig.3, below). These increases in phosphorylation correlated with hCG-induced increases in cyclic AMP and testosterone production, and the ED_{50} of hCG for testosterone stimulation was 0.5 pM (fig.3). Incubation of Leydig cells with Bt_2cAMP caused phosphorylation of 10 bands that were identical to those stimulated by hCG. Of the cyclic AMP-dependent phosphorylated bands observed in the Leydig cell extracts, 5 proteins with M_r 21 000, 25 000, 29 000, 37 000 and 66 000) corresponded to the hCG-stimulated protein bands observed in the intact Leydig cells stimulated by hCG or Bt_2cAMP.

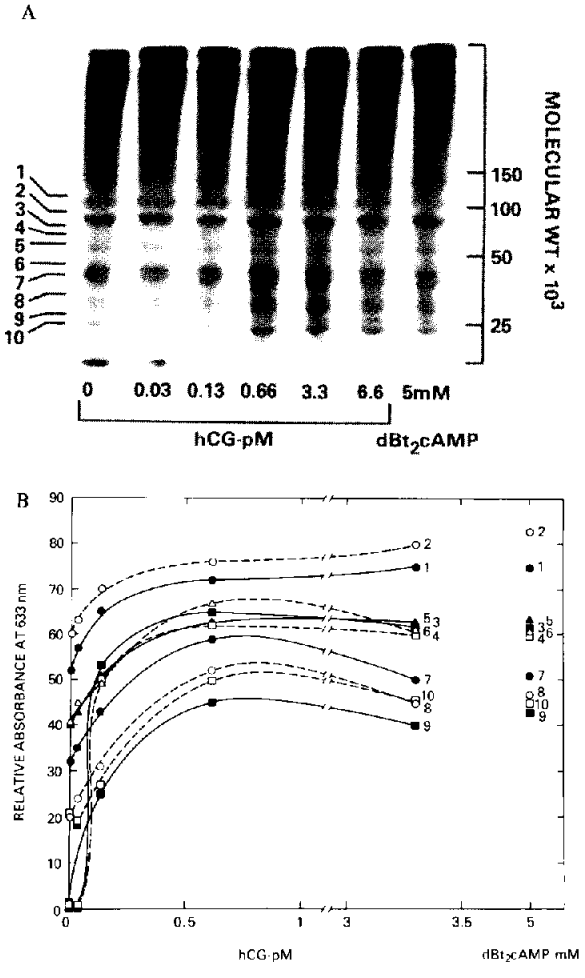

Fig.2. Phosphorylation of protein substrates in hCG-stimulated Leydig cells. (Above) Autoradiogram of SDS–polyacrylamide gel electrophoresis of Leydig cells prelabeled with [^{32}P]orthophosphoric acid and stimulated with increasing concentrations of hCG or 5 mM dibutyryl cAMP (Bt_2cAMP) for 10 min. The hCG-induced phosphorylated bands are labeled 1–10 on the right. (Below) Plot of densitometric measurements of the autoradiogram shown above.

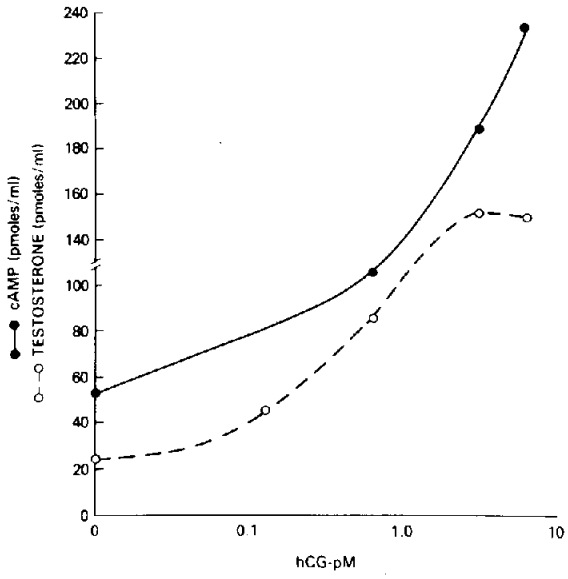

Fig.3. Cyclic AMP and testosterone production during gonadotropic stimulation of Leydig cells in vitro for 30 min.

The nature of the phosphorylated substrates was evaluated by treatment of ^{32}P-labeled Leydig cell extracts with trypsin and other enzymes. Trypsin treatment caused complete disappearance of all phosphorylated bands from the gels, whereas neither deoxyribonuclease I nor ribonuclease caused any change in the ^{32}P-labeled bands. In addition, no significant differences in total phosphate incorporation were observed after trichloroacetic acid precipitation, followed by sequential heating and washing to remove RNA and phospholipids.

Further resolution of the hCG-induced phosphorylation products was performed by two-dimensional

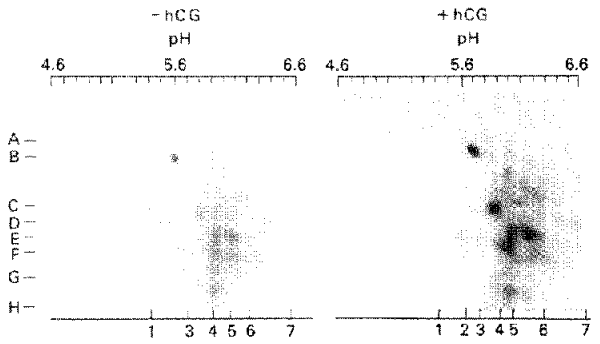

Fig.4. Two-dimensional gel electrophoresis of phosphorylated proteins in control (left) and hCG-stimulated Leydig cells (right). Cells were incubated with 0.66 pM hCG for 10 min.

gel electrophoresis, of which a representative autoradiogram is shown in fig.4. Ten discrete protein bands with M_r 22 000–105 000 were observed. The hormone-induced increases in phosphorylation were 90–700% (fig.5,6), being greatest in proteins with M_r 30 000 with pI 5.35 (G_1), 36 000, pI 5.35 (F_1), 42 000, pI 5.5 (E_2), 38 000, pI 5.95 (E_4), 42 000, pI 6.0 (E_5), 42 000, pI 6.2 (E_6), 58 000, pI 5.75 (C_4) and 105 000, pI 5.7 (B_3). Seven of the bands in which hormone-stimulated phosphorylation was demonstrated by double-dimension gel electrophoresis were of identical M_r with bands detected on one-dimensional gel electrophoresis after stimulation by hormone and Bt_2cAMP.

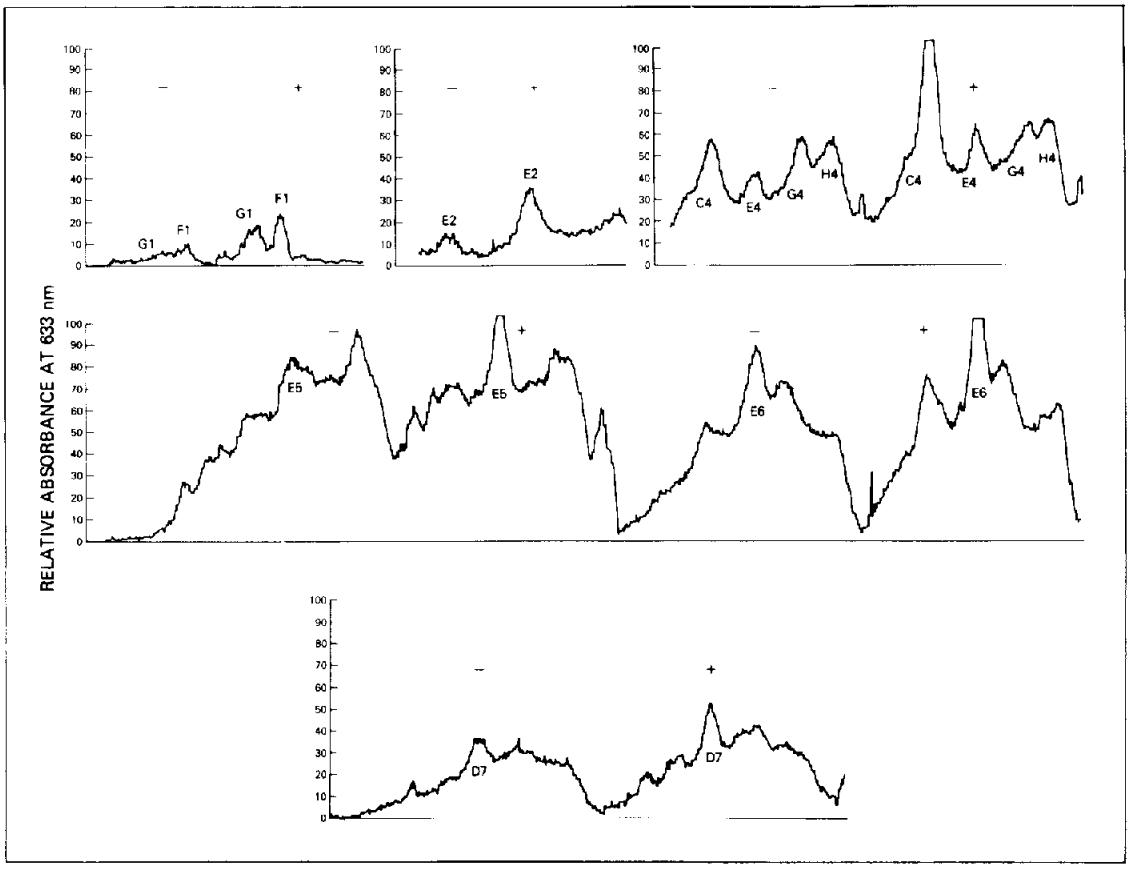

Fig.5. Densitometric tracings of individual phosphorylated proteins derived from experiments shown in fig.5, from control Leydig cells, and from cells stimulated with hCG. In each panel, the control tracing is followed by the tracing of the corresponding stimulated preparation. The densitometer was calibrated to 100% absorbance with the most heavily labeled protein (A_2, M_r 105 000) shown in the right panel, and the scale indicates relative absorbance.

4. Discussion

These studies have demonstrated that at least 10 Leydig cell proteins are rapidly phosphorylated during gonadotropin action, and that the same proteins are labeled during stimulation with Bt_2cAMP. Also, the experiments with Leydig cell extracts revealed that several of the proteins showing cyclic AMP-dependent phosphorylation were coincident with those observed in intact cells during hormone action. In the Leydig cell, increases in cyclic AMP and stimulation of cyclic AMP-dependent protein kinase have been observed within 1–2 min after hCG stimulation in vitro [1–5]. Therefore, it is not surprising that prominent increases in phosphorylation were detectable at 5 and 10 min. After hormone-induced increases of intracellular cyclic AMP, or addition of cyclic AMP derivatives to cell extracts, very rapid cyclic AMP-dependent phosphorylation was observed within 30 s in the majority of protein substrates, with only 4 proteins reaching their maximum at 5 min. This is also consistent with the timing of the steroidogenic process, where pregnenolone increases after gonadotropin action has a lag time of about 10 min and the increases in testosterone follow at 15 min [10].

These studies present the first demonstration that rapid, dose-related phosphorylation is induced by gonadotropin in a number of proteins of the Leydig cell. Hormone-dependent phosphorylation of a single Leydig cell protein of M_r 57 000 has been observed [11]; this protein corresponds to one of those described here, and could have a role in the acute actions of gonadotropin. Recent studies in the adrenal gland have demonstrated phosphorylation and/or dephosphorylation of 7 proteins with M_r 14 000–210 000 [12], at least two of which were similar to those observed here. In contrast with the testis, during ACTH or cyclic AMP-induced phosphorylation of adrenal extracts, several proteins were dephosphorylated after initial rapid phosphorylation. Since both types of steroidogenic cells possess similar early enzymatic pathways, and presumably a common mechanism of activation of these enzymes, similarities in the phosphorylation patterns may be expected to occur. Also, in the adrenal, ACTH stimulates the phosphorylation of a rapidly turning over mitochondrial protein that appears to influence the conversion of cholesterol to pregnenolone [13]. In addition, cholesterol esterase can be phosphorylated and activated by cyclic AMP-dependent protein kinase [14,15], though the 2–3-fold stimulation was less than the several-fold increase in steroidogenesis observed during ACTH action [15].

The relationship between steroidogenic enzymes and their activation through cyclic AMP-dependent protein kinase during trophic hormone action can now be investigated by parallel mapping of phosphorylated proteins and enzyme activity. In the testis, such a correlation could prove difficult since the Leydig cells comprise only a minor fraction of the testicular tissue. Also, regulatory enzymes such as mitochondrial cytochrome P450 [16], cholesterol esterase [17] and 3 methyl-glutaryl CoA reductase [18] are present in extremely small quantities. However, the identification of the proteins shown to be phosphorylated during gonadotropin action represents an important goal in the elucidation of the mechanisms by which hormones control steroid biosynthesis and secretion.

In summary, the post-cyclic AMP/protein kinase events evoked by gonadotropin action in the Leydig cell were investigated by electrophoretic analysis of phosphorylated protein substrates. These studies have demonstrated that both hCG and dibutyryl cyclic AMP induce phosphorylation of multiple proteins in the Leydig cell. These events are correlated with hCG-stimulated increases in cyclic AMP and testosterone production, indicating that the steroidogenic response to LH receptor occupancy is mediated by activation of the cyclic AMP/protein kinase/phosphorylation sequence during hormonal stimulation.

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