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hCG INCREASED PHOSPHORYLATION OF PROTEINS IN PRIMARY CULTURES OF PORCINE LEYDIG CELLS

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Phosphorylation of endogenous proteins after hCG stimulation was studied in primary cultures of purified porcine Leydig cells. The hormone increases the phosphorylation of at least 6 proteins and more specifically of a protein D of MW of about 90 K. The increased phosphorylation of this protein was observed within 10 minutes after addition of the hormone, at a time when stimulation of testosterone production was only slightly increased, and was maximum between 20 and 30 minutes. hCG dose-response curves for both testosterone production and protein D phosphorylation were parallel. Leydig cell proteins phosphorylation was also observed after stimulation with 8-Bromo-cAMP.

LH stimulation of testis steroidogenesis involves several steps (1-3) including binding of the hormone to the plasma membrane receptors, followed by stimulation of the production of cyclic AMP and activation of protein kinase. It has been suggested that phosphorylation of specific cell proteins might mediate the steroidogenic effect of the hormone (4).

In rat Leydig cell suspensions several authors have shown a lutropin induced phosphorylation of endogenous proteins and correlated it with an increase of cyclic AMP and testosterone production (5, 6). A major disadvantage in the use of rat interstitial tissue is that the isolated cell population is only 10-40 % Leydig cells (7). Moreover, it has been recently shown (8) that the Leydig cells from adult rats are formed of two different populations, one of which has a very poor response to hCG despite the fact that the number and the affinity of LH/hCG receptors are similar in both. Therefore in this model it is almost impossible to precise the cell origin of the phosphorylated proteins and to establish a correlation with steroidogenesis. Recent studies of this laboratory have shown that interstitial cells isolated from pig testis present several advantages when compared to the rodent model : the number of LH/hCG receptors per cell is higher and the Leydig cells represent more than 50 % of the total cell population (9). Moreover these cells cultured for several days in a chemically defined medium keep their ability to respond to hCG in terms of cAMP and steroid production (10).

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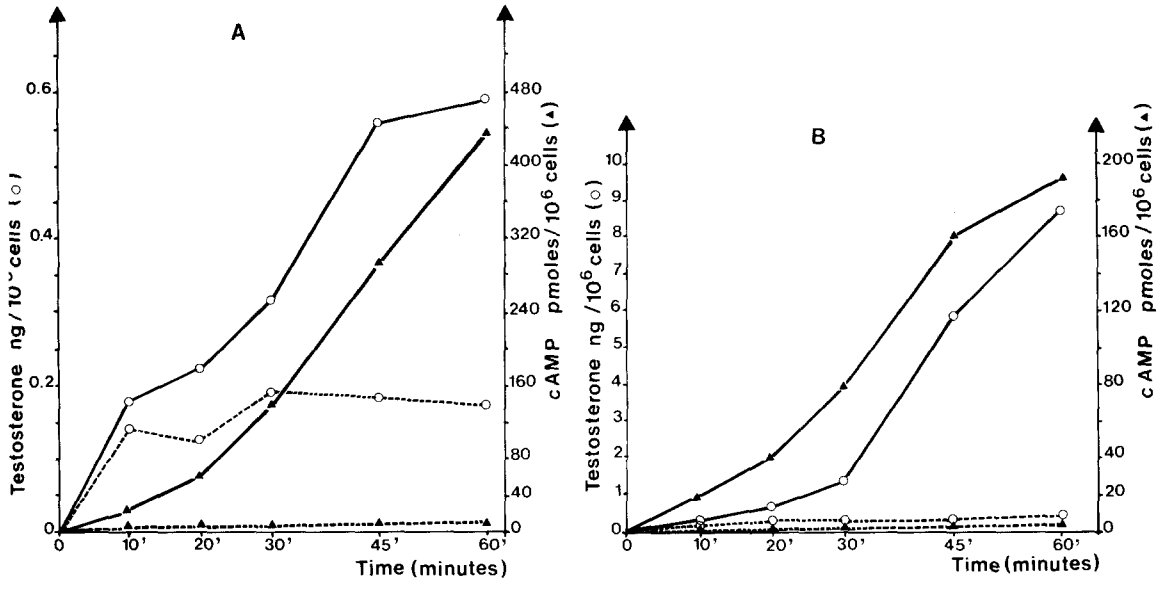

Figure 1. Time course of the effects of hCG (66 ng/ml) on testosterone (O) and cAMP (▲) productions by non purified Leydig cells (left pannel, A) and purified Leydig cells (right pannel, B). The productions of testosterone (O---O) and cAMP (▲---▲) in the absence of the hormone are also shown. Both purified and non purified Leydig cells were used after 48 hours of culture as described in Material and Methods (Note the difference in the scale for testosterone production in pannels A and B).

Therefore we have studied the effects of hCG on phosphorylation and steroidogenesis using primary cultures of either total interstitial cells or cells purified by a Percoll gradient (11). In this case the population of Leydig cells is homogenous as checked by the measurement of hCG receptors, the steroidogenic response and the 3β-hydroxysteroid deshydrogenase staining.

MATERIAL AND METHODS

Gonadotrophine chorionique "Endo" pregnyl was from Organon, Saint-Denis (France). 8-bromoadenosine 3'5' cyclic AMP monophosphate (8Br. cAMP) was purchased from Sigma. [^{32}P]Pi (specific activity 20 mCi/mg) was purchased from Saclay, France. 3-isobutyl-1-methylxanthine (MIX) was from Sigma.

Porcine testes were decapsulated, minced and treated by collagenase, as described by Mather et al (9). Purified Leydig cells were obtained using a continuous Percoll gradient, and the details of the methods will be published elsewhere (11). This preparation gives more than 90 % pure Leydig cells as controlled by 3β-hydroxysteroid deshydrogenase staining.

Cells were grown in Falcon flasks (25 cm^2) (3 to 5 x 10^6 cells) at 37°C in an atmosphere of 5 % CO_2 in medium HAM F12/DME 1/1 in the presence of sodium bicarbonate 1.2 mg/l, Hepes 10 mM, gentamycine 20 µg/l, mycostatin 10 µg/l, fetal calf serum 0.1 %, insulin 5 µg/ml, transferrin 5 µg/ml, EGF 10 ng/ml, α-tocoferol 10 µg/ml, and the medium is changed every day. After 48 hours of culture the medium was removed and the cells washed with saline at 37°C. The cells were then incubated in Puck's medium, without phosphate, containing Hepes 10 mM, calcium chloride 1 mM and 150 µCi of ^{32}P/ml at 37°C for 30 minutes. After this preincubation time, hCG at various concentrations (indicated in the Figures) was added and the stimulation stopped at different times 10, 20, 30, 45, and 60 minutes. Control incubations without hCG were run in parallel. At the end of the incubation, 1 ml of medium was removed and poured in

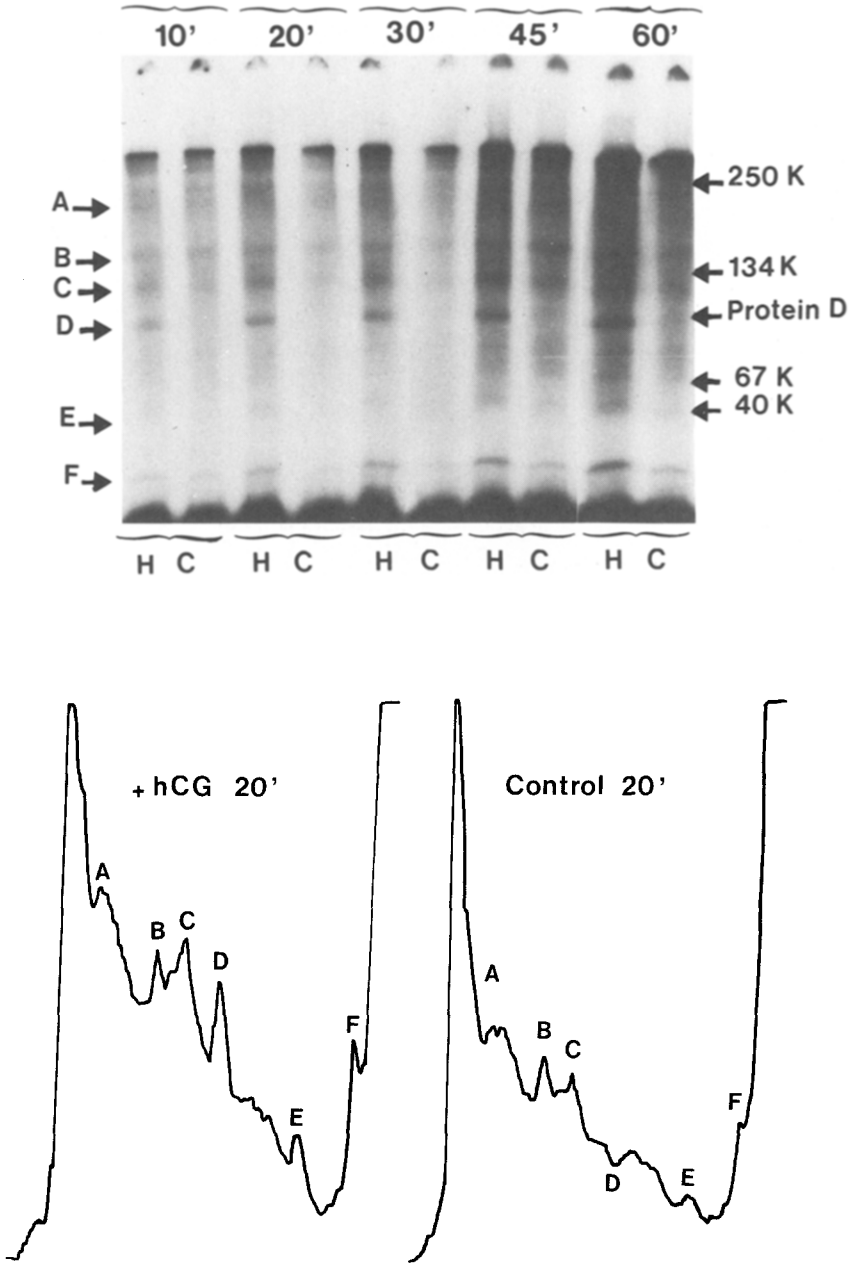

Figure 2. Time course of the protein phosphorylation of purified Leydig cells in the presence (H), or absence (C) of hCG (66 ng/ml).
Upper pannel. Autoradiographs of SDS polyacrylamide gels electrophoresis. The standards used are catalase (250 K), bovine serum albumin dimer (134 K), bovine serum albumin monomer (67 K), ovalbumin (40 K).
Lower pannel. Densitometric tracing of the autoradiographies at 20 minutes (cells were cultured for 48 hours, as indicated in Methods).

3 ml of ethanol for cyclic AMP measurement and the remaining was stored at -20°C for testosterone measurement.

The incorporation of ^{32}P was stopped by 5 washes with PO_4 medium 0.2 M and the cells dissolved in boiling SDS 1 % (1 or 2 ml according to the number of cells in the

incubation). Electrophoresis of the dissolved samples was carried out in slab gels as previously described (12) at pH 7.2 using acrylamide at a concentration of 11 % in system J 3561 described by Neville and Glossman (13) in the presence of 0.1 % SDS in the upper reservoir buffer. The slab-gels were dried and exposed to Kodak films NS 2T. Autoradiographs were scanned using a photometer integrator PHI 6 Vernon.

Testosterone from the medium was extracted with ethyl-ether. Then the ether was washed 6 times with water in order to completely eliminate the ^{32}P, and evaporated. Testosterone was measured by radioimmunoassay (14). Cyclic AMP was measured by radioimmunoassay (15) after acetylation (16).

RESULTS

In preliminary experiments using non purified Leydig cells, hCG induced within 10 minutes the phosphorylation of a protein (MW of about 90 K) (data not shown).

However, since the hCG-induced testosterone (which is the specific marker of Leydig cell function) production by purified Leydig cells was several times higher than that of non purified cells as shown in Figure 1, all further experiments were performed using purified Leydig cells. It must be noted however that the hCG-induced cAMP production by non purified cells was higher than that of purified cells. This might be due to the contamination of hCG by FSH-like activity, and to the presence of Sertoli cells in non purified preparations of Leydig cells.

The time course effects of hCG-induced protein phosphorylation of purified cells is shown in Fig. 2 (upper pannel). hCG stimulates the phosphorylation of at least 6 proteins, as it is clearly shown by the densitometric tracing of the autoradiographs (Fig. 2, lower pannel).

In order to obtain more quantitative data the peak height analysis of these proteins was performed at different times using the densitometric tracing of the autoradiographs. The phosphorylation of the six proteins increases with time in both control and hCG-treated cells (Fig. 3). However, due to the fact that the level of phosphorylation of protein D in control conditions is lower than that of all the other proteins except protein E, the effect of the hormone on the phosphorylation of protein D is much higher than the one observed for the other proteins. The MW of this protein D was estimated by comparing its mobility to the mobilities of known MW standards (Fig. 2) and is about 90 K. Maximum effect of hCG on phosphorylation of protein D is observed between 20 and 30 minutes. All these results were confirmed in three other independent experiments.

These effects of hCG on the phosphorylation of Leydig cells proteins and more specifically on protein D are probably mediated by cyclic AMP since the incubation of the purified Leydig cells in the presence of 8Br-cAMP (1 mM) gives similar results to those obtained with the hormone (Fig. 4).

hCG dose-response curves for protein D phosphorylation, cAMP and testosterone productions is shown in Figure 5. The lowest dose of hCG able to induce a significant increase of both testosterone production and protein D phosphorylation is 1 ng, and maximum effect for both was obtained with 66 ng. It is particularly interesting to note that the dose-response curve for protein phosphorylation and

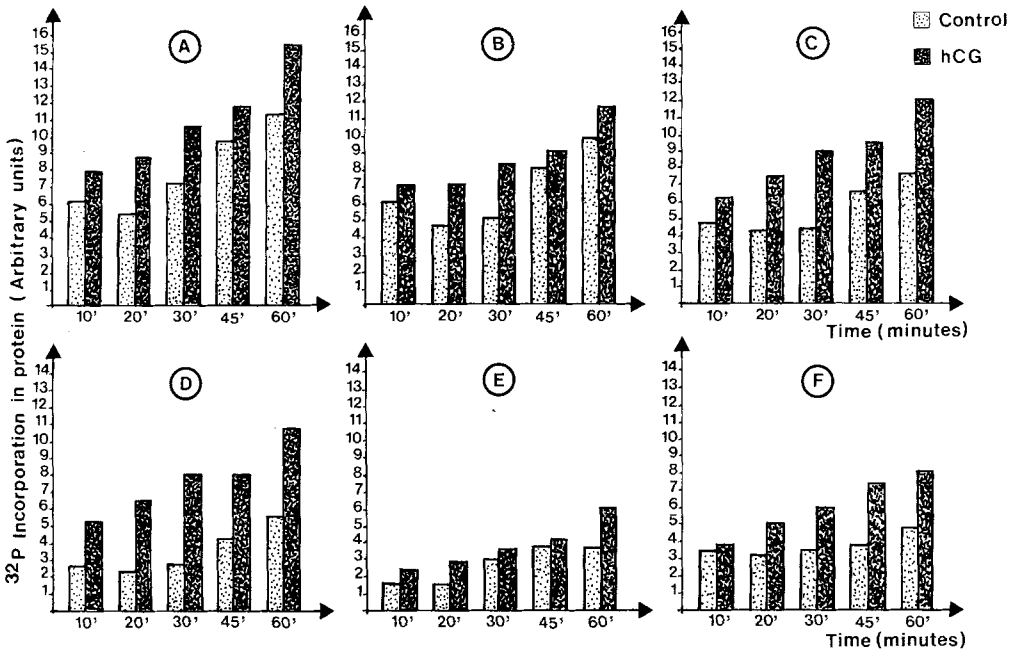

Figure 3. Time course of the phosphorylation of proteins A, B, C, D, E, F. The absorbance value (peak heights) given in arbitrary units is obtained from the densitometric scanning of the autoradiographs shown in Fig. 2 (darker bars represent the incorporation in presence of hCG, 66 ng/ml).

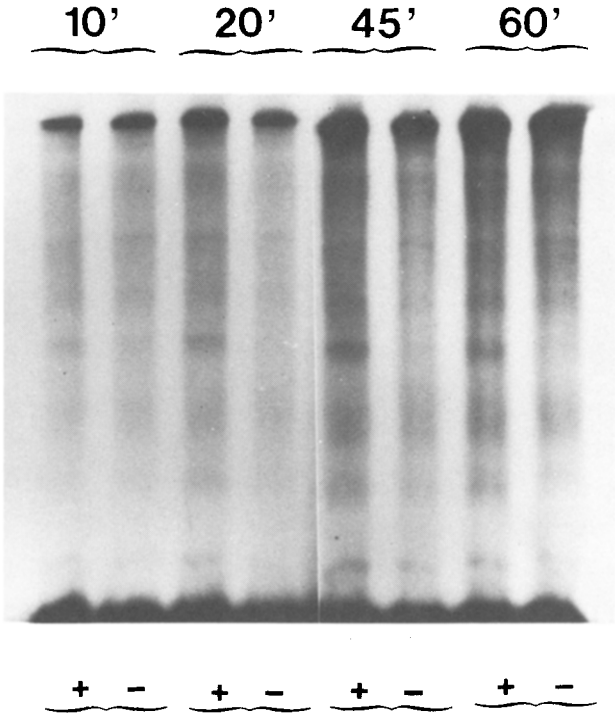

Figure 4. Time course of the protein phosphorylation of purified Leydig cells in the absence (–) or presence (+) of 1 mM 8Br cAMP. The cells were cultured for 48 hours as indicated in Methods.

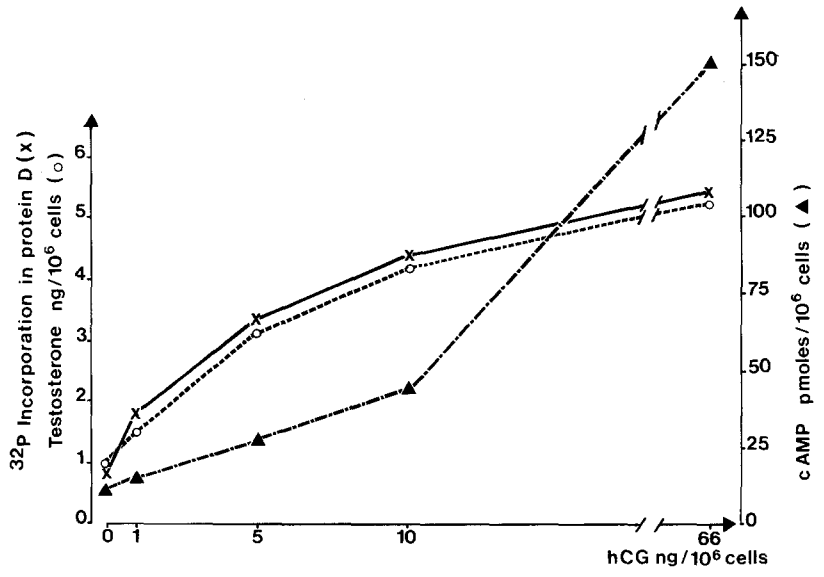

Figure 5. hCG dose response curve for production of cAMP (▲), testosterone (O) and phosphorylation of protein D (X) (arbitrary units). Leydig cells after 48 h of culture were preincubated with ^{32}P for 30 minutes (as described in Material and Methods) and then for 30 minutes in the presence of the amounts of hCG indicated, and 0.1 mM MIX. Protein D phosphorylation was quantified by densitometric scanning of the autoradiographs.

testosterone production are very similar to each other but are different from that of cAMP production.

DISCUSSION

From our data obtained with purified Leydig porcine cells, we have shown that hCG increases the phosphorylation of several proteins, but more specifically of a protein with an approximate MW of 90 K, the effect being reproduced by cAMP. This phosphorylation was achieved with hormone concentrations within the steroidogenic range. Moreover while there is a similarity between the phosphorylation of protein D and the production of testosterone at the different amounts of hCG used, the stimulation of cAMP production by the hormone is different. This discrepancy between the hCG dose response curves for cyclic AMP production and phosphorylation of protein D is similar to that reported for cAMP production and protein kinase activation in Leydig cells (7, 17), in adrenal cells (18), and in luteal cells (19). Our results clearly indicate that both testosterone production and protein phosphorylation are increased by low physiological doses of the hormone. Using luteal cells Darbon et al (19) have also shown that LH increases the phosphorylation of several proteins, one of which has a same MW as our protein D. Whether or not the phosphoproteins mentioned in our study are the mediators of the steroidogenic effect of hCG in porcine Leydig cells remains to be demonstrated. Further work to characterize the nature of these phosphoproteins and to precise their subcellular localization could help to elucidate their role.

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