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hCG-INCREASED PHOSPHORYLATION OF PROTEINS IN PRIMARY CULTURE OF LEYDIG CELLS : FURTHER CHARACTERIZATION

A. Dazord[+], A. Genot[++], D. Langlois-Gallet[+], C. Mombrial[+], F. Haour[+] and J.M. Saez[+]

+ INSERM U. 162, Hôpital Debrousse, 69322 Lyon Cedex 05, France
++ UER Lyon-Nord, Laboratoire de Biochimie Médicale, 69373 Lyon Cedex 08 France

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Previous studies using monodimensional gel electrophoresis of total cell proteins have shown that hCG was able to increase the phosphorylation of at least 6 proteins in cultured porcine Leydig cells. By using subcellular fractionation and 2D gel electrophoresis, we now show that most of these proteins, whose phosphorylation was increased by hCG, are basic nuclear proteins. In addition, hCG also increases the phosphorylation of microsomal proteins in particular ribosomal protein S6. Moreover no phosphoproteins were detected in the cytosol.

We have recently shown (1) that in primary cultures of porcine Leydig cells, hCG was able to increase the phosphorylation of at least 6 proteins and more specifically, of a protein D of apparent MW of 90 K. In the present study, we have tried to localize these phosphoproteins ; the subcellular fractionationation suggests that most of them are both in the nucleus and in the ribosomes, but are absent in the cytosol.

MATERIALS AND METHODS

Gonadotrophine chorionique "Endo" pregnyl was from Organon, Saint-Denis (France). [^{32}P] Pi (specific activity 20 mCi/mg) was purchased from Saclay, France.

Porcine testes were decapsulated, minced and treated by collagenase, as described by Mather et al (2). Cells were grown in 75 cm^2 Falcon flasks (10 to 15 x 10^6 cells) at 33°C in an atmosphere of 5 % CO_2 in Ham's F12/DME (1/1) medium 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, α - tocoferol 10 µg/ml.

After 48 hours of culture the medium was removed and the cells washed with saline at 33°C. The cells were then incubated for 30 minutes, in Puck's medium (without phosphate) containing Hepes 10 mM, calcium chloride 10 mM and 150 µCi of ^{32}P/ml. After this preincubation, hCG at various concentrations was added and the stimulation stopped at different times from 10 to 60 minutes. Control incubations without hCG were run in parallel. Most experiments described in this study were performed using a 30 minutes hCG incubation time, at a concentration of 50 ng/ml.

Isolation of subcellular components. At the end of the incubation period with or without the hormone, the incorporation of [^{32}P] into proteins was stopped by 5 washes with phosphate buffer 0.1 M pH 7, rinsed with saline, scrapped with a disposable cell scraper, then lysed for 10 minutes at 4°C in a Potter Elvejehm Teflon glass homogenizer (A A) (50 strokes at maximum speed) centrifuged at 2000xg for 15 minutes. The pellet obtained was referred as "nuclear preparation". The efficiency of the lysis and the integrity of the nuclei were checked by microscope examination using toluidine blue.

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In some experiments, the 2000xg supernatant was further centrifuged at 10,000xg followed by a 100,000xg centrifugation. The resulting pellet was referred to as "microsomal preparations".

Ribosome preparation. The homogenate of Leydig cells was made 0.3 M in KCl, 3 mM in $MgCl_2$, 1 % in Triton X100 and 1 % in sodium deoxycholate. After stirring for 5 minutes at 4°C, centrifugation was performed at 700xg for 5 min. in a SW60 rotor, then at 7000xg in the same rotor for another 5 min. The post mitochondrial supernatant was finally layered over 3 ml of 1 M sucrose containing 50 mM ethanolamine, 75 mM KCl, 5 mM $MgCl_2$, then centrifuged for 14 h at 200,000xg in a 50 rotor.

Extraction of ribosomal proteins. Ribosomal proteins were extracted from the whole pellet using acetic acid. The methods are detailed elsewhere (3, 4). The resulting proteins were dialyzed against 1N acetic acid and lyophilized.

Polyacrylamide gel electrophoresis. Monodimensional gel electrophoresis was performed as previously described (5). The 2000xg and the 100,000xg pellets are resuspended in sodium dodecylsulfate 1 %, then sonicated 3 times for 10 seconds each with a MSE sonicator.

Two-dimensional gel electrophoresis for the nuclear proteins were performed as described elsewhere (6) using, in the first dimension, either an acidic system with migration toward the cathode, or a basic system with migration toward the anode. The former resolves proteins whose pHi is higher than 4, while the latter resolves proteins whose pHi is lower than 8. The second dimension separating gel was 11 % acrylamide, containing SDS 0.1 %, as described in our previous work (6).

Two-dimensional gel electrophoresis of the ribosomal proteins. Ribosomal proteins were analyzed by 2D gel electrophoresis followed by autoradiography according to our previous publication (7). Radioactive spots were identified according the nomenclature proposed (8).

RESULTS

Phosphorylation pattern of the nuclear preparations. Figure 1 shows the comparative pattern of phosphorylation in the whole cell fraction as well as in the nucleus of these cultured in the presence or absence of the hormone. It is clear that protein D (90 K) is present in the nucleus and to a lesser extent B (MW = 150 K), C (MW = 125 K), protein E (MW = 40 K) and F (MW = 25 K). hCG increases the phosphorylation of these 5 proteins. However, protein D is the most important, while protein E is barely detected.

In order to obtain a better resolution of the nuclear proteins, these were submitted to 2D polyacrylamide gel electrophoresis. The first dimension was run at acidic pH, and the second in the presence of SDS. Fig. 2 shows that in the nuclei from control cells, there were at least 7 phosphoproteins, the most important being protein D. Following hCG treatment the phosphorylation of all these proteins was increased particularly that of protein D and F.

When the first dimension was performed at a basic pH (8.6), and the migration is toward the anode, no phosphoproteins were detected (data not shown). This means that these proteins have a pHi higher than 8, and are positively charged at pH 8.6. This is confirmed by the fact that, after an extraction with 2 M NaCl which extracts only acid proteins, we were not able to detect any phosphoproteins. Protein F whose MW was estimated to be 25 K is probably histone $H1^0$, although as seen below, it is also detected in the microsomes.

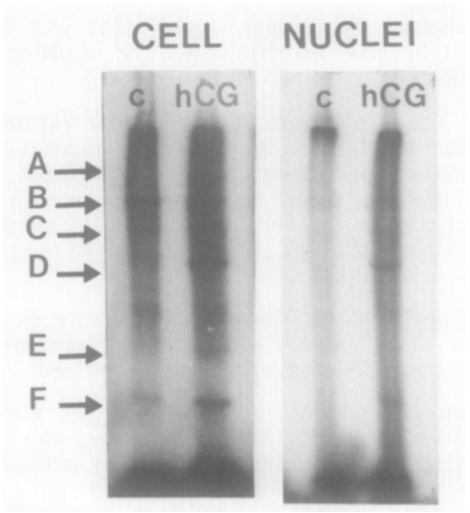

Figure 1. Phosphorylation pattern of nuclear proteins as compared to the 1 % SDS extracted cell preparations. Proteins are detected by autoradiography as previously described (1).
C = control conditions - h = hCG treatment (50 ng/ml for 30 min.).

Phosphorylation pattern of the microsomal preparations and of the ribosomes. The profile of phosphoproteins obtained in the microsomes was quite different from that observed in the nuclei (fig. 3).

Protein D is not present, while protein B (150 K), E (40 K) and F are present. Only phosphorylation of B and E was increased by hCG.

The 2D gel of the ribosomes (fig. 4) shows that there are two major ribosomal proteins or microsome-associated phosphorylated proteins in control and after hCG stimulation. One of them is S_6, and its phosphorylation is clearly increased by hCG. In addition there is another high MW protein whose phosphorylation is increased by hCG which is probably protein B (150 K). The third phosphoprotein detected in the ribosome might be protein E. Its phosphorylation is only slightly increased by hCG and it does

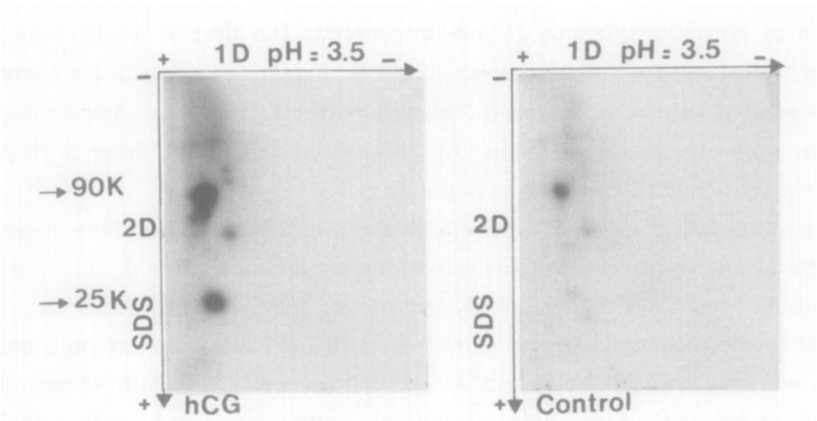

Figure 2. 2D gel electrophoresis of the nuclear pellet.

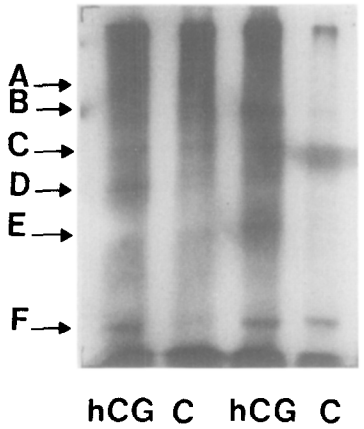

Figure 3. Phosphorylation pattern of the microsomal proteins as compared to the nuclear proteins.
C = control - h = hCG treatment (as in figure 1).

not correspond to any known ribosomal proteins (8). Lastly, in at least 4 experiments in which the cytosol (100,000xg supernatant) was analyzed no phosphoproteins could be detected (data not shown).

Incorporation of radioactive aminoacids into proteins. Since the phosphorylation of S_6 could be implicated (references in 9), it was considered relevent to study whether in our model, the hCG-induced phosphorylation of S_6 was accompanied by an increase in protein synthesis (fig. 5). The results shown in this figure clearly indicate that even if the cells are incubated for 30 minutes or 4 hours with hCG, this hormone was unable to induce a significant increase in protein synthesis.

DISCUSSION

It is well known that the steroidogenic effect of several hormones : ACTH in the adrenal (10), LH and hCG in the gonads (11) is induced by cAMP. Since the only

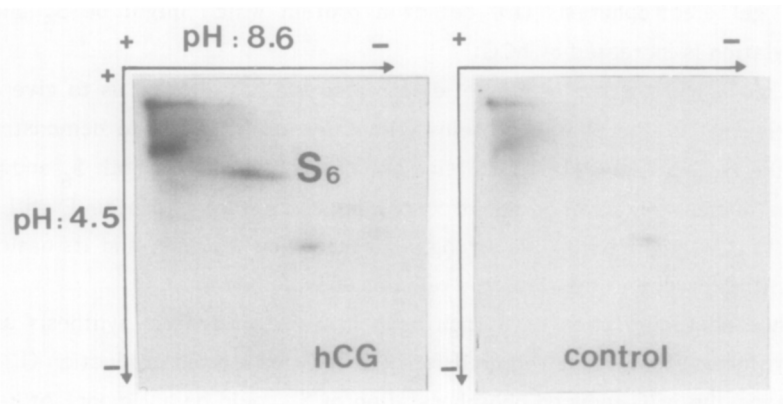

Figure 4. Phosphorylation pattern of the ribosomal proteins.

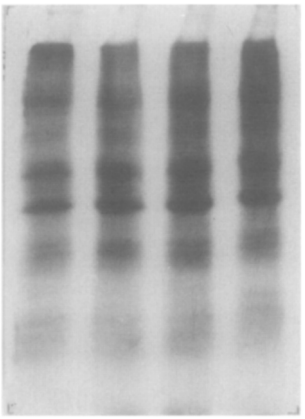

Figure 5. Incorporation of [^{3}H] leucine in Leydig cell proteins under control conditions (C) and after hCG treatment. Electrophoresis was performed in 11 % acrylamide gel, containing SDS.

known mediators of cAMP in gonads are the cAMP-dependent protein kinases, it has been postulated that phosphoproteins could be implicated in the activation of steroidogenesis. However, up to now, very little is known about the nature and the role of such phosphoproteins.

In a previous work (1) using primary cultures of pig Leydig cells, we have shown that hCG increased the phosphorylation of several proteins and that there was a good correlation between hCG-induced increase phosphorylation of protein D and testosterone production.

The present results demonstrate that the phosphoproteins stimulated by hCG are mainly localized in the nucleus and ribosomes. In this respect our data are in good agreement with the results reported on rat tumour Leydig cells (10-14). These studies using 1D gel electrophoresis (13) detect a protein which might be S_6 and whose phosphorylation is increased by hCG.

In our work, the use of 2D gel electrophoresis has allowed us to give a better characterization of the phosphoproteins stimulated by hCG and to demonstrate that most of the hCG-phosphorylated proteins are basic, and include both S_6 and probably $H1^0$ in the nucleus. However, a doubt concerning the nature of protein F still persists. The fact that it might be $H1^0$ is strongly suggested by the MW, and its basic nature. However, its presence in the ribosome remains unexplained.

Since phosphorylation of S_6 has been involved in protein synthesis and since protein synthesis seems to be required for hCG-induced steroid production (12-14), our data showing the hCG-induced phosphorylation of S_6 could be an important key in our understanding of the steroidogenic effect of hormones. Although we were unable to

detect acute stimulation of protein synthesis by hCG under our experimental conditions, we cannot exclude the possibility that hCG-induced modifications of specific proteins exist. One unexpected finding was the absence of phosphorylation of cytosolic proteins by hCG-stimulation. This is contradictory to what is reported in rat tumour Leydig cells (13). It is unlikely that our results may be due to a complete dephosphorylation of cytosolic proteins, since all the cytosolic preparations were performed in the presence of 10 mM NaF. Another possibility for the discrepancy between our results and those of Bakker (12-14) is the fact that the results obtained in the rat model, and especially in tumor cells (15), cannot be extrapolated to other normal species. Indeed, the pig is probably closer to man than the rat (15) as far as regulation of steroidogenesis is concerned.

In summary, our results underline the coexistence of increased phosphorylation of nuclear and ribosomal proteins and testosterone production by normal pig Leydig cells, following hCG stimulation. However, the nature and the role of most of these proteins are unknown and further studies are required in order to determine their functions.

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REFERENCES

1. Gonzalez-Martinez, A., Benahmed, M., Bommelaer, M.C., Haour, F., Saez, J.M., and Dazord, A. (1982) Biochem. Biophys. Res. Commun. **105**, 334-340.
2. Mather, J.P., Saez, J.M., and Haour, F. (1981) Steroids **38**, 35-44.
3. Hardy, S.J.S., Kurland, G., Voynow, P., and Mora, G. (1969) Biochemistry **8**, 2897-2905.
4. Sherton, C.C., and Wool, I.G. (1974) Mol. Gen. Genet. **135**, 97-112.
5. Dazord, A., Gallet de Santerre, D., and Saez, J.M. (1981) Biochem. Biophys. Res. Commun. **98**, 885-891.
6. Dazord, A., Langlois-Gallet, D., and Saez, J.M. (1984) J. Steroid Biochem., in press.
7. Genot, A., Reboud, J.P., Cenatiempo, Y., and Cozzone, A.J. (1978) FEBS Lett. **86**, 103-107.
8. McConkey, E.H., Bielka, H., Gordon, J., Castick, S.M., Lin, A., Ogata, K., Reboud, J.P., Traugh, J.A., Traut, R.R., Warner, J.R., Welfle, H., and Wool, I.G. (1979) Mol. Gen. Genet. **169**, 1-6.
9. Traugh, J.A. (1981) in Biochemical Actions of Hormones, Vol. VIII, pp. 167-208. Academic Press, New York (G. Litwak ed.).
10. Saez, J.M., Morera, A.M., and Dazord, A. (1981) Adv. Cyclic Nucleot. Res. **14**, 563-579.
11. Saez, J., Haour, F., Tell, G., Gallet, D. and Sanchez, P. (1978) Mol. Pharmacol. **14**, 1054-1062.
12. Bakker, G.H., Hoogerbrugge, J.W., Rommerts, F.F.G., and Van der Molen, H.J. (1981) Biochem. J. **198**, 339-346.
13. Bakker, G.H., Hoogerbrugge, J.W., Rommerts, F.F.G., and Van der Molen, H.J. (1982) Biochem. J. **204**, 809-815.
14. Bakker, G.H., Hoogerbrugge, J.W., Rommerts, F.F.G., and Van-der-Molen, H.J. (1983) FEBS Lett. **161**, 33-36.
15. Saez, J.M., Benahmed, M., Reventos, J., Bommelaer, M.C., Mombrial, C., and Haour, F. (1983) J. Steroid Biochem. **19**, 375-384.