Effect of Lutropin on Phosphorylation of Endogenous Proteins in Testis Leydig Cells

CORRELATION WITH TESTOSTERONE PRODUCTION

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The effect of lutropin on phosphorylation of endogenous proteins in testis Leydig cells was investigated, by incubating purified Leydig cells with lutropin and [^{32}P]P_i followed by sodium dodecyl sulphate/polyacrylamide-slab gel electrophoresis of the [^{32}P]phosphoproteins. The radioactivity of the proteins was quantified by densitometry of the radioautograms obtained. The following results were obtained. 1. Lutropin increased the amount of ^{32}P incorporated into three proteins (A, B and C) with apparent mol.wts. of 14 300, 57 000 and 77 600 respectively. 2. The increase in incorporation of ^{32}P into these proteins was detectable within 5 min, reaching a maximum in approx. 20 min. 3. The ^{32}P incorporated into protein B (but not proteins A and C) was significantly increased with 0.1 and 1.0 ng of lutropin/ml. Incorporation of ^{32}P into all three proteins was significantly increased with 10 ng of lutropin/ml, reaching a maximum with 100 ng/ml. 4. Testosterone production was significantly increased with 1 ng of lutropin/ml, and between 10 and 1000 ng/ml the degree of stimulation of testosterone production and incorporation of ^{32}P into proteins A, B and C was similar. 5. Cyclic AMP production was significantly increased with 10 ng of lutropin/ml and had not reached a maximum with 1000 ng/ml. 6. In Leydig cells isolated from hypophysectomized rats 3 h after injection of choriogonadotropin *in vivo*, phosphoproteins with the same molecular weights as proteins A, B and C were found. No further increases in incorporation of ^{32}P into these proteins were obtained when lutropin was added to the Leydig cells *in vitro*. 7. Dibutyl cyclic AMP (but not follitropin or testosterone) also stimulated the incorporation of ^{32}P into proteins A, B and C in Leydig cells.

Cyclic AMP-dependent protein kinases in testis Leydig cells can be activated by lutropin (luteinizing hormone, LH) *in vitro* (Cooke & van der Kemp, 1976; Podesta *et al.*, 1976a), and there is a dose-dependent correlation between activation of protein kinase and stimulation of production of testosterone (Cooke *et al.*, 1976). This is in contrast with cyclic AMP production, which is undetectable with doses of lutropin that result in one-half to maximum stimulation of steroidogenesis (Catt & Dufau, 1973; Moyle & Ramachandran, 1973; Rommerts *et al.*, 1973; Cooke *et al.*, 1976). These results suggested that, in testis Leydig cells, activation of protein kinase, the only known mechanism by which cyclic AMP acts in eukaryotic cells (see review by Rubin & Rosen, 1975), is a more sensitive parameter for lutropin stimulation than measuring cyclic AMP itself. These previous experiments were carried out by using exogenous substrates (histones) for the protein kinases. The present investigation was undertaken to determine the nature of the endogenous substrates for

protein kinases in Leydig cells and the kinetics and lutropin-dose dependency of their phosphorylation. This has been achieved by incubating purified rat-testis Leydig cells with lutropin and [^{32}P]P_i followed by radioautography of the phosphoproteins after electrophoresis on sodium dodecyl sulphate/polyacrylamide slab gels.

Materials and Methods

Sheep lutropin (N.I.H.-LH-S18; 1.03 i.u./mg) and follitropin (N.I.H.-FSH-S11; 1.15 i.u./mg) were gifts from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. Human choriogonadotropin was from Organon, Oss, The Netherlands. 3-Isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. [^{32}P]P_i (100–130 $\mu\text{Ci}/\text{mg}$ of P) was from The Radiochemical Centre, Amersham, Bucks., U.K. Adult Wistar rats, substrain R-Amsterdam (14–16 weeks old, 200–250 g), kept under controlled light

(14h light and 10h dark) and temperature (20–22°C) conditions, were used in this study.

Leydig-cell suspensions from rat testes were prepared and purified by centrifugation through Ficoll and dextran solutions as previously described by Janszen *et al.* (1976a). Leydig cells were preincubated (to increase the sensitivity to lutropin; Cooke *et al.*, 1976, 1977) in Krebs–Ringer solution (without phosphate), pH 7.4 (Umbreit *et al.*, 1964), containing 0.1% bovine serum albumin (fraction V) for 2h under an atmosphere of O₂/CO₂ (19:1). Lutropin, 3-isobutyl-1-methylxanthine (0.25mM) and [³²P]P_i (25μCi) were added together or at different times during incubations carried out for different time periods as indicated in the text. Each incubation contained approx. 3 × 10⁶ nucleated cells/ml, incubation volume 0.1 ml. Incubations were stopped by adding 2–3 ml of Krebs–Ringer buffer (containing 0.15M-KH₂PO₄ and 20mM-NaF) at 0°C. The cells were centrifuged at 100g for 10min and the pellets were resuspended in fresh buffer and sonicated (5s at 20kHz with an amplitude of 5μm at 0°C). Trichloroacetic acid (10%, w/v) was added and, after centrifugation (1000g), the pellet was washed further with trichloroacetic acid (0.5ml; twice), ethanol (70%, v/v; 0.5ml) and chloroform/methanol (1:1, v/v; 0.5ml). Finally the pellets were dissolved in Tris/glycerol buffer [0.05M-Tris/HCl/10% (v/v) glycerol, pH 6.8, containing 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol] and heated at 100°C for 2min. Electrophoresis was carried out in 10 and 15% (w/v) discontinuous sodium dodecyl sulphate/polyacrylamide slab gels as described by Laemmli (1970). After electrophoresis, the gels were fixed in methanol/water/acetic acid (5:4:1, by vol.) overnight. The gels were then stained with 1% Amido Black in 7% (v/v) acetic acid for 30min and destained by several washings with methanol/water/acetic acid (30:63:7, by vol.). The gels were dried (at 70–80°C) on a Bio-Rad gel-slab dryer under continuous heating. After drying, the gels were exposed to Kodak X-ray film RP14. The radioautograms obtained were scanned with a Vitatron TLD 100 densitometer. The correlation between the amount of radioactivity in the gel and the densitogram of the exposed X-ray film was tested with radioautograms of known increasing amounts of radioactivity. To correct for inter-sample variation in total radioactivity, the ratio of the peak height of each band divided by the peak height of a band with mol.wt. approx. 40000 (which was shown to be unaffected by the different incubation conditions used) was calculated. This ratio was used for a quantitative evaluation of the amount of ³²P incorporated into the protein bands. The molecular weights of the proteins were calculated by comparison with the mobilities of known molecular-weight standards: cytochrome *c* (11 700), ovalbumin (43 000), chymotrypsin (25 700), catalase (60 000) and phosphorylase A (90 000).

Separate incubations with Leydig cells were carried out to determine the production of cyclic AMP and testosterone as described previously (Cooke *et al.*, 1976). For statistical analysis Student's *t* test for correlated data was used. Differences were considered significant when *P* < 0.05 (one-tailed).

Results

Phosphorylation of Leydig-cell proteins and effect of lutropin

Incubation of Leydig cells with [³²P]P_i resulted in phosphorylation of several proteins as demonstrated by radioautography of the cell extracts after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1). The radioactivity of the proteins most probably reflects labelled phosphate esterified to threonine

Fig. 1. Radioautogram of ³²P-labelled proteins from Leydig cells

Leydig cells were preincubated for 2h in a phosphate-free Krebs–Ringer buffer and then separate incubations were carried out with different amounts of lutropin in the presence of 0.25mM-3-isobutyl-1-methylxanthine for 2h. [³²P]P_i (25μCi) was added 1h before the end of the incubation period. The incubations were stopped (see the Materials and Methods section for details) and the washed trichloroacetic acid precipitates subsequently obtained were subjected to electrophoresis on 10% and 15% discontinuous sodium dodecyl sulphate/polyacrylamide gels. The radioautogram shown is from a typical experiment and was obtained by exposing the dried gel to X-ray film for 4 days. The mobilities of known standard molecular-weight markers are indicated on the left-hand side of the radioautogram. A, B and C refer to the three proteins (mol.wts. 14 300, 57 000 and 77 600 respectively) in which the incorporation of ³²P was increased by lutropin. For details of the experimental procedure see the Materials and Methods section.

Fig. 2. Time course of effect of lutropin on incorporation of ^{32}P into Leydig-cell proteins A (■), B (▲) and C (●). Leydig cells were preincubated for 2 h in phosphate-free Krebs-Ringer buffer and then separate incubations were carried out for 3 h in the presence of 0.25 mM-3-isobutyl-1-methylxanthine. $^{32}\text{P}\text{P}_i$ (25 μCi) was added 1 h before the end of the incubation period. Lutropin (100 ng/ml) was added to the incubations at various times as indicated in the Figure before the end of the incubation period. Electrophoresis and radioautography were carried out as described in the legend to Fig. 1 and in the Materials and Methods section. The results shown (from duplicate incubations), expressed as percentage of the maximum incorporation of ^{32}P , are typical of three experiments.

or serine residues, because lipid extraction, treatment with hot or cold trichloroacetic acid, or with 1 M-NaOH at 4°C, did not decrease the radioactivity of the isolated proteins. Treatment with 1 M-NaOH at 37°C from 1 to 24 h in the presence of albumin (1 mg) completely removed the ^{32}P phosphate. In the absence of added albumin, the Leydig-cell proteins (approx. 10^6 cells) were unstable in the presence of NaOH.

Addition of lutropin to the incubation medium of the Leydig cells increased the ^{32}P incorporated into three bands (referred to as proteins A, B and C) which had average mol.wts. of $14\,300 \pm 200$, $57\,000 \pm 300$ and $77\,600 \pm 700$ (means $\pm$ S.E.M., $n = 6-8$) respectively (Fig. 1). The rate of incorporation was rapid; detectable increases were observed within 5 min of addition of lutropin. Maximum incorporation of ^{32}P was achieved in proteins B and C within approx. 20 min, whereas protein A was phosphorylated more slowly (Fig. 2). No decrease in incorporation of ^{32}P occurred during 180 min incubation with lutropin. In these experiments the cells were incubated for 2-3 h; lutropin was added at various times before the end of the incubation period. In separate experiments in which the cells were incubated for 120 min with lutropin, $^{32}\text{P}\text{P}_i$ was added for 5-120 min before the end of the incubation period; no qualitative difference in the proteins phosphorylated was found.

Fig. 3. Lutropin dose-response curves for incorporation of ^{32}P into Leydig-cell proteins A (■), B (▲) and C (●). The results (means $\pm$ S.E.M., $n = 6$), which are from three separate experiments carried out in duplicate, are expressed as percentage of maximum incorporation of ^{32}P obtained with 100 ng of lutropin/ml. See the legend to Fig. 1 and the Materials and Methods section for further experimental details.

Fig. 4. Lutropin dose-response curves for testosterone (●) and cyclic AMP (■) production in Leydig cells.

Production of testosterone and cyclic AMP was measured in the same series of experiments described in the legend to Fig. 3. Incubations were carried out for 2 h in the presence of 0.25 M-3-isobutyl-1-methylxanthine with different amounts of lutropin as indicated. Testosterone production is given as a percentage of that obtained with 1000 ng of lutropin/ml. The results (means $\pm$ S.E.M., $n = 6$) are from three separate experiments carried out in duplicate. See the Materials and Methods section for further experimental details.

Lutropin dose-response relationships for phosphorylation of protein and production of testosterone and cyclic AMP

Incubations with Leydig cells were carried out with different amounts of lutropin, and the amount of ^{32}P incorporated into proteins A, B and C was measured (Fig. 3). The results are expressed as percentage of maximum stimulation obtained with 100 ng of lutropin/ml. Without addition of lutropin, incorporation of ^{32}P into proteins A and C was 50-60% of

maximum and into protein B was approx. 25% of maximum. The amount of ^{32}P incorporated into protein B (but not proteins A and C) was significantly increased with 0.1 and 1.0 ng of lutropin/ml, from $26.7 \pm 3.4\%$ (without added lutropin) to 34.4 ± 2.3 and $35.6 \pm 3.0\%$ respectively ($P < 0.025$ and < 0.005 respectively; means $\pm$ S.E.M., $n = 5$). The amount of ^{32}P incorporated in all three proteins was significantly increased with 10 ng of lutropin/ml, reaching a maximum with 100 ng/ml. In the same series of experiments, production of testosterone and cyclic AMP was measured. For comparative purposes the production of testosterone was calculated as a percentage of that obtained with 1000 ng of lutropin/ml, which gave a near-maximum production (see also Cooke *et al.*, 1976). With 0.1 ng of lutropin/ml no increase in testosterone production was detected, but with 1.0 ng/ml a significant increase occurred compared with the basal production (from $8.3 \pm 2.8\%$ to $19 \pm 3.9\%$; $P < 0.05$; mean $\pm$ S.E.M., $n = 6$). With 10 ng of lutropin/ml large increases occurred. Between 10 and 1000 ng/ml the degree of stimulation of incorporation of ^{32}P into proteins A, B and C and of production of testosterone was similar (Figs. 3 and 4). In contrast, and in agreement with previous work, a significant increase in cyclic AMP production above that in the absence of added lutropin did not occur until 10 ng of lutropin/ml was added ($P < 0.05$) and maximum production was not reached with 1000 ng/ml. Also, a significant ($P < 0.01$) decrease in production of cyclic AMP occurred when 0.1 ng of lutropin/ml was added compared with the production in the absence of added hormone (1.78 ± 0.81 and 3.68 ± 1.32 pmol/ 10^6 cells respectively) (means $\pm$ S.E.M., $n = 6$).

Effect of choriogonadotropin in vivo on phosphorylation of Leydig cells from hypophysectomized rats

The effect of administration *in vivo* of choriogonadotropin on phosphorylation of the Leydig-cell proteins *in vitro* was also investigated. Rats were hypophysectomized 1 day before intravenous (tail-vein) administration of choriogonadotropin (100 i.u.) or 0.9% (w/v) NaCl. Then 3 h later the animals were decapitated and Leydig cells were prepared and incubated with and without lutropin for 2 h (no preincubation). In the Leydig cells from the NaCl-injected hypophysectomized rats, lutropin (100 ng/ml) stimulated incorporation of ^{32}P into three protein bands with the same molecular weights as those obtained from Leydig cells from normal intact rats. In the absence of added lutropin, the ^{32}P incorporated into these proteins (A, B and C) was $52 \pm 1\%$, $33 \pm 11\%$ and 53% respectively (means $\pm$ range of duplicate incubations) of that obtained in the presence of lutropin, i.e. the 'basal' amount of ^{32}P incorporated was not lower in hypophysectomized animals than in intact males. In the Leydig cells isolated from the

hypophysectomized rats injected with choriogonadotropin no further increase in incorporation of ^{32}P into these proteins was obtained when lutropin was added to the cells; without added lutropin the amount of ^{32}P incorporated was $104 \pm 0.5\%$, $83 \pm 6\%$ and 93% (means $\pm$ range of duplicate incubations) of that in the presence of added lutropin. The amount of ^{32}P incorporated in the cells from the choriogonadotropin-treated animals was similar to that obtained in the lutropin-stimulated cells from the controls. In the Leydig cells obtained from the control hypophysectomized rats, the production of cyclic AMP and testosterone was $3.4 \pm 0.6\%$ and $6.4 \pm 0.5\%$ respectively (means $\pm$ ranges of duplicate experiments) of those obtained in the presence of lutropin. In the hypophysectomized rats treated with choriogonadotropin, the production of cyclic AMP and testosterone was 91.5 ± 1.5 and $84.5 \pm 2.5\%$ respectively (means $\pm$ range of duplicate experiments) of those found in the presence of added lutropin. These results indicate that 3 h after injection of choriogonadotropin (plus $1\frac{1}{2}$ h for the preparation of the cells) the cells were still fully activated by the choriogonadotropin.

Specificity of lutropin effects

No effect of follitropin (100 ng/ml) or testosterone (50 ng/ml) on incorporation of ^{32}P into the Leydig-cell proteins could be found after incubation of the Leydig cells for 2 h. Dibutyryl cyclic AMP (1 mM) increased incorporation of ^{32}P into proteins with the same molecular weight as those obtained after stimulation with lutropin [control values were 44 ± 11 , 70 ± 20 and $59 \pm 11\%$ of dibutyryl cyclic AMP-stimulated samples for proteins A, B and C respectively (means $\pm$ ranges of duplicate incubations)].

After incubation of [^{32}P]P_i with preparations containing small amounts of Leydig cells (6%) (obtained by collagenase treatment of rat testes without further purification), phosphoproteins with the same molecular weights as proteins A, B and C were detectable. In such preparations, smaller increases of incorporation of ^{32}P into proteins A and B were obtained with both lutropin (100 ng/ml) and dibutyryl cyclic AMP (control values were 70–80% of those for stimulated samples) compared with the purified preparations containing 60% Leydig cells. Similarly, in incubations with seminiferous tubules, phosphoproteins with the same molecular weights as proteins A, B and C were detectable, but no effect of lutropin, follitropin, testosterone or dibutyryl cyclic AMP on incorporation of ^{32}P could be demonstrated.

Discussion

In this study it has been shown that lutropin stimulates the phosphorylation in testis Leydig cells of three endogenous proteins with apparent mol.wts. of

14300, 57000 and 77600 (proteins A, B and C respectively) as determined by their electrophoretic mobilities on sodium dodecyl sulphate/polyacrylamide gels. This effect of lutropin was also found with dibutyryl cyclic AMP, but not with follitropin or testosterone. No effect of lutropin, follitropin or testosterone on incorporation of ^{32}P in seminiferous tubules could be detected, indicating that lutropin via cyclic AMP specifically stimulates phosphorylation of proteins A, B and C in testis Leydig cells.

The proteins A, B and C were also phosphorylated without the addition of exogenous lutropin; the amount of ^{32}P incorporated, compared with the maximum incorporated in the presence of lutropin, was not decreased by hypophysectomy. It is possible that this phosphorylation was either independent of lutropin or was due to bound endogenous lutropin even when the cells are isolated from rats 1 day after hypophysectomy. The phosphoproteins remain phosphorylated for long periods of time, as shown by the time studies after stimulation with lutropin *in vitro* and after stimulation with choriogonadotropin *in vivo*. Alternatively, more than one protein may be present in each protein band, whose phosphorylation may not be cyclic AMP-dependent.

Increases in the amount of ^{32}P incorporated into the proteins A, B and C were shown to be dependent on the dose of lutropin; maximum stimulation occurred with doses of lutropin that were similar to those required for near-maximum stimulation of testosterone synthesis. With low amounts of lutropin (0.1 and 1.0 ng/ml) there was a small but significant stimulation of the phosphorylation of protein B. The lowest amount of lutropin that gave a detectable stimulation of testosterone synthesis was 1.0 ng/ml. In a previous study (Cooke *et al.*, 1976) it was shown that the minimum doses of lutropin required to stimulate testosterone and protein kinases were the same. Thus it may be concluded that with both exogenous and endogenous substrates there is a correlation between the dose-dependent lutropin-stimulated protein kinase activation and steroidogenesis. These results are in contrast with those reported by Podesta *et al.* (1976b), who found that low concentrations of choriogonadotropin stimulated testosterone production in Leydig-cell preparations without causing a change in activation of protein kinase or production of cyclic AMP. Doses of choriogonadotropin that resulted in maximum production of testosterone had to be added before increases in protein kinases and cyclic AMP production could be detected. One possible explanation for the discrepancies between the results of these two studies is the different methodology used to isolate the protein kinases before assay. In previous studies (Cooke & van der Kemp, 1976; Cooke *et al.*, 1976) it was shown that the use of NaCl in the isolation medium for testis tissues should be avoided, as this causes

dissociation of the protein kinase subunits, a decrease in total enzyme activity and an apparent loss of sensitivity to lutropin. Although Podesta *et al.* (1976b) showed that NaCl had little effect on their protein kinase activity, their enzyme was assayed after elution with 0.5 M-NaCl from DEAE-Sephadex columns. The activity measured by these authors may therefore be only a part of that originally present in the Leydig cells.

In agreement with previous work (Catt & Dufau, 1973; Moyle & Ramachandran, 1973; Rommerts *et al.*, 1973; Mendelson *et al.*, 1975; Cooke *et al.*, 1976), it was again found that changes in cyclic AMP did not reflect changes in testosterone concentrations with different doses of lutropin. Even in the presence of a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, it was found that a small but significant decrease in the production of cyclic AMP occurred with 0.1 ng of lutropin/ml when compared with the control. It is possible therefore that any increases that may have occurred with 1 ng of lutropin/ml may have been masked by metabolism of the cyclic AMP.

The nature and possible functions of the proteins A, B and C is still unknown. Reports from our laboratory (Janszen *et al.*, 1976b, 1977) have shown that there are two other Leydig-cell proteins, of apparent mol.wts. 33000 and 21000, that may have a function in the mechanism of lutropin action on steroidogenesis. The first of these proteins has a short half-life (about 11 min) and its synthesis is not under the influence of lutropin. The synthesis of the second protein is induced by lutropin and has a half-life of more than 30 min. In the present study no phosphorylation of these proteins after stimulation with lutropin could be detected in Leydig cells isolated from normal and hypophysectomized rats. This indicates that the phosphorylated forms of these proteins are not involved in their possible actions on steroidogenesis.

To conclude, it has been found that lutropin specifically stimulates the phosphorylation of three endogenous proteins in testis Leydig cells. The amount of ^{32}P incorporated with different doses of lutropin approximately parallels the increases in testosterone biosynthesis, thus suggesting that these proteins may be involved in the control of steroidogenesis via stimulation of cyclic AMP and protein kinase activation.

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