Subcellular localization of LH-dependent phosphoproteins and their possible role in regulation of steroidogenesis in rat tumour Leydig cells

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Stimulation of rat tumour Leydig cells with LH resulted in phosphorylation of 7 proteins of 17, 22, 24, 33, 43, 57 and 76 kDa, and in dephosphorylation of a single protein of 20 kDa. The subcellular localization of these LH-dependent phosphoproteins in combination with effects of inhibitors of microfilament formation and protein synthesis, suggest that phosphoproteins of 20, 43 and 76 kDa present in the cytosol may be involved in the action of microfilaments, whilst phosphoproteins of 24 and 33 kDa present in microsomes may be involved in specific protein synthesis.

Lutropin regulation *Steroidogenesis* *Leydig cell* *Protein phosphorylation*

1. INTRODUCTION

Hormonal stimulation of steroidogenic cells involves cAMP-dependent phosphorylation of specific proteins. The increases in phosphorylation of specific phosphoproteins and steroid production show similar dose–response relationships [7,9,11,13] and similar kinetics [4,9,13].

Little is known about the subcellular localization and possible function of these phosphoproteins. Authors in [13] described the subcellular localization of some specific phosphoproteins in adrenal tissue. We have reported earlier the absence of phosphoproteins in mitochondria, the presence of a single phosphoprotein of M_r 17000 in nuclei and of 4 LH-dependent phosphoproteins of M_r 22000, 24000, 33000 and 57000 in the post-mitochondrial supernatant [4]. It was also shown, that the M_r 33000 phosphoprotein was similar to ribosomal protein S6 [5].

We now describe a further analysis of the subcellular localization and possible function of the LH-dependent phosphoproteins in tumour Leydig cells.

2. MATERIALS AND METHODS

Chemicals used, procedures for isolation of tumour Leydig cells, incubation conditions, SDS–polyacrylamide gel electrophoresis of phosphorylated proteins, incorporation of ^{3}H- and ^{14}C-labelled amino acids into proteins have in essence been described in [4,5]. [4,5-^{3}H]Leucine (58 Ci/mmol) and [5-^{3}H]uridine (5 Ci/mmol) were purchased from The Radiochemical Centre, Amersham (Bucks). Actinomycin D was obtained from Boehringer (Mannheim). Cytochalasin B was from Sigma (St Louis MO); 5-cholestene-3β,25-diol (25-hydroxycholesterol) was from Steraloids (Wilton NH). Experiments were performed generally with 1–2 × 10^6 cells preincubated at 32°C for 1 h, and incubated for another hour with or without LH/MIX.

2.1. *Subcellular fractionation*

Microsomes (199000 × g_{av} pellet) and cytosol (199000 × g_{av} supernatant) were isolated from the post-mitochondrial supernatant prepared as in [4]. The medium for homogenization of tumour

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Leydig cells [10] contained: 10 mM KCl, 10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 2 mM dithiothreitol, 20 mM NaF. After homogenization of tumour Leydig cells with a Dounce glass homogenizer, the osmolarity of the medium was adjusted to that of 0.15 M NaCl by addition of 0.1 volume of a buffer containing: 1 mM KCl, 200 mM Tris–HCl (pH 7.5), 30 mM $MgCl_2$, 20 mM dithiothreitol, 20 mM NaF. For subfractionation of the postmitochondrial supernatant a Beckman SW 40-rotor (40000 rev./min – 199000 × g_{av}) for 3 h 40 min, or a Beckman Airfuge (100000 rev./min – 132000 × g_{av}) for 17 min was used.

2.2. *Electrophoresis of phosphorylated proteins*

SDS–polyacrylamide gel electrophoresis of phosphorylated proteins was performed with equal portions of the proteins present in the microsomal and cytosol fractions. Details on measurement of LH effects on protein phosphorylation have been published elsewhere [4].

2.3. *Effects of actinomycin D*

The effects of actinomycin D (10 μg/ml) were investigated on incorporation of 2 μCi [^{3}H]uridine or [^{3}H]leucine into macromolecules, and on control and LH-dependent pregnenolone production.

Table 1

Effect of actinomycin D on incorporation of [^{3}H]uridine and [^{3}H]leucine into macromolecules, and on LH-dependent pregnenolone production

Cellular activity	% Decrease
Incorporation of [^{3}H]uridine	97 ± 3 (3)
Incorporation of [^{3}H]leucine	4 ± 11 (3)
LH-dependent pregnenolone production	4 ± 5 (6)

Tumour Leydig cells attached to a Petri dish were preincubated for 2 h, following by incubation for 1 h at 32°C in the presence of [^{3}H]uridine, [^{3}H]leucine or inhibitors of pregnenolone metabolism, with or without actinomycin D (10 μg/ml). The average % decrease was calculated from individual values obtained within each experiment. Results are means ± SD with the number of different cell preparations in parentheses. Basal and LH-dependent pregnenolone production (in ng.h^{-1} mg protein^{-1}) were 185 ± 89 and 622 ± 400 (mean ± SD; n = 6)

Table 2

Subcellular localization of lutropin-dependent phosphoproteins in microsomal and cytosol fractions of tumour Leydig cells

Molecular mass	Fraction	
	Microsomes	Cytosol
20000	6% ± 6	94% ± 6
22000	4% ± 4	96% ± 4
24000	94% ± 7	6% ± 7
33000	95% ± 7	5% ± 7
43000	8% ± 5	92% ± 5
57000	32% ± 4	68% ± 4
76000	5% ± 7	95% ± 7

Microsomes and cytosol were isolated, and equal portions of the fractions were separated with SDS–polyacrylamide gel electrophoresis (see fig.1). For all the proteins indicated, the LH effects on protein phosphorylation were measured from the peak heights in densitograms obtained from microsomes and cytosol. The sum of the LH/MIX-induced peak in cytosol and microsomal fractions was taken as 100%. The distribution of the phosphoproteins over the two subcellular fractions was calculated from the relative contributions of the LH/MIX-induced protein phosphorylation. Results are means ± SD (from 4 different subcellular fractionations)

2.4. *Effects of cytochalasin B*

The effects of cytochalasin B (50 μM) were investigated on pregnenolone production and phosphorylation of proteins under basal and LH/MIX-stimulated conditions.

3. RESULTS AND DISCUSSION

It was previously shown [4] that all LH-dependent phosphoproteins (except the M_r 17000 protein) are localized in the post-mitochondrial supernatant. The M_r 17000 protein was localized in nuclei and this protein may play a role in the hormonal regulation of nuclear activities. We have evaluated therefore the possible role of RNA synthesis in the short-term regulation of steroidogenesis in tumour Leydig cells. Addition of actinomycin D to tumour Leydig cells caused an almost complete inhibition of incorporation of [^{3}H]uridine, with no inhibition of incorporation of [^{3}H]leucine or LH-dependent pregnenolone pro-

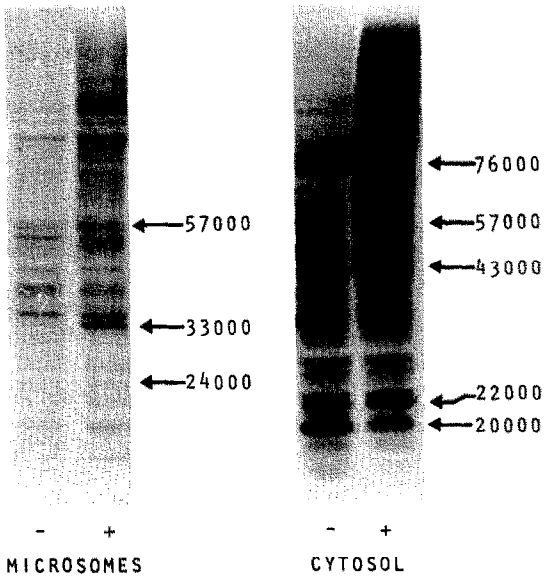

Fig.1. Autoradiogram showing the localization of LH-dependent phosphoproteins in microsomal and cytosol fractions of tumour Leydig cells. Tumour Leydig cells (15×10^6) were incubated in the presence of $^{32}P_i$ without (−) or with (+) LH/MIX. Microsomes and cytosol were obtained using the Airfuge (see section 2). Arrows indicate LH-dependent phosphoproteins with their molecular masses.

duction (table 1). These results suggest that the M_r 17000 protein is probably not involved in the short-term regulation of steroid production. The M_r 17000 protein may play a role, however, in long-term tropic effects of LH.

The distribution of the remaining post-mitochondrial phosphoproteins over the microsomal and cytosol fraction was also investigated. The M_r 24000 and 33000 proteins were exclusively recovered in the microsomal fraction, whereas the 20000, 22000, 43000 and 76000 proteins were present in the cytosol (table 2). The pattern of the LH-independent phosphoproteins in microsomes and cytosol was completely different without indications for mutual contamination (fig.1). The presence of the M_r 57000 protein in microsomes (for 30%) and in cytosol (for 70%) may therefore represent a true double localization of this protein.

Owing to the fractionation procedure, the presence of M_r 43000 and 76000 proteins could be shown without difficulty in the cytosol fraction, whereas this was almost impossible in extracts from intact cells. The intensity of the 43000 protein was higher after isolation of the cytosol fraction, using an Airfuge (centrifugation time 17 min) than after using a standard ultracentrifuge (centrifugation time 3 h 40 min). This difference could be due to a shorter exposure time of the isolated phosphoproteins to phosphatases released after the homogenization procedure.

The presence of the 76000 and 30000 protein in the cytosol and the hormone-dependent phosphorylation of the 76000 protein coinciding with the dephosphorylation of the 20000 protein

Table 3

Effect of cytochalasin B (50 μM) on pregnenolone production in tumour Leydig cells

	No cytochalasin B ($ng.h^{-1}.mg$ $protein^{-1}$)	With cytochalasin B ($ng.h^{-1}.mg$ $protein^{-1}$)	% Inhibition
Control	57.5 ± 26.9	25.5 ± 16.7	58.3 ± 9.7
LH/MIX	421.8 ± 93.9	229.0 ± 49.8	45.7 ± 3.2
25-OH-chol	983.0 ± 231.0	848.5 ± 210.0	14.0 ± 1.7

Tumour Leydig cells attached to a Petri dish were preincubated for 1 h, followed by incubation for 1 h at 32°C. Incubations were with or without LH/MIX or with 25-hydroxycholesterol (25-OH-chol; 32 μM). The average % inhibition was calculated from the individual values obtained within each experiment. Statistical significance of the inhibition by cytochalasin B was $p < 0.01$ (paired t-test) under all conditions tested. Results are means ± SD obtained in 3 different experiments with duplicate incubations

suggest that the 76000 and 20000 phosphoproteins may be similar to myosin light-chain kinase and myosin light-chain, respectively [1,6]. Moreover, the LH-dependent phosphoprotein of M_r 43000 in the cytosol and actin share the same molecular mass (e.g., [16]). It has been suggested that microfilaments play a role in hormone-dependent steroid production [8,12,17]. Phosphorylation of microfilament proteins may represent an essential step in this process. Effects of cytochalasin B (50 μM; cf. [3,15]) on protein phosphorylation and steroid production were investigated to test this hypothesis. No effects on protein phosphorylation were detected (not shown), whereas control and LH-dependent pregnenolone production were inhibited to about 50% (table 3). The specificity of cytochalasin B effects was investigated by measuring the hormone-independent cholesterol side-chain cleavage activity in the presence of 25-hydroxycholesterol [2,14,18]. A small but significant inhibition of the side-chain cleavage activity was found (table 3), indicating that for full cholesterol side-chain cleavage activity either intact microfilaments are essential or cytochalasin B has non-specific effects. In any case, the inhibitory effects of cytochalasin B on hormone-dependent pregnenolone production are much larger, which supports the notion that microfilaments are essential for regulation of steroid production.

The presence of M_r 24000 and 33000 proteins in the microsomes suggests that these proteins may play a role in specific protein synthesis (cf. [5]). However, thus far no LH-induced protein has been identified whose action can explain the rapid effect of LH on increased steroid production, and the precise function of the microsomal phosphoproteins remains to be demonstrated.

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