PHOSPHOPROTEINS AND REGULATION OF STEROIDOGENESIS IN RAT TUMOUR LEYDIG CELLS

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PROEFSCHRIFT

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CONTENTS

Appendix Papers (continued)

IV. G.H. Bakker, J.W. Hoogerbrugge, F.F.G. Rommerts & H.J. van der Molen: "Possible functions of LH-dependent phosphoproteins and protein synthesis in the regulation of cholesterol side-chain cleavage activity in rat tumour Leydig cells". Submitted for publication to Molec. Cell. Endocr.

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LIST OF NON-STANDARD ABBREVIATIONS

Ci	–	Curie, unit of radioactivity
Da	–	Dalton, unit of molecular mass
DNA	–	deoxyribonucleic acid
eIF	–	eukaryotic initiation factor
FAD	–	flavin adenine dinucleotide
(m)RNA	–	(messenger) ribonucleic acid
NADPH	–	reduced nicotinamide adenine dinucleotide phosphate
R	–	rectus, right-hand side
RTO	–	rapidly-turning-over
S	–	Svedberg, unit of sedimentation
SDS	–	sodium dodecyl sulphate
SNCF	–	Société Nationale des Chemins de fer Français
TGV	–	train à grande vitesse, très grande vitesse

LIST OF TRIVIAL NAMES

cholesterol	–	5-cholestene-3β-ol
cholesterol side-chain cleavage enzyme	–	total of three proteins performing side-chain cleavage of cholesterol yielding pregnenolone and isocaproaldehyde (cf. section 2.2)
corticosterone	–	4-pregnene-11β,21-diol-3,20-dione
corticotropin	–	ACTH, adrenocorticotropic hormone
cyclic AMP	–	adenosine cyclic-3',5'-monophosphate
cyclic GMP	–	guanosine cyclic-3',5'-monophosphate
dibutyryl cyclic AMP	–	N^6-2'-O-dibutyryl adenosine cyclic-3',5'-monophosphate
follitropin	–	FSH, follicle-stimulating hormone
25-hydroxycholesterol	–	5-cholestene-3β,25-diol
isocaproaldehyde	–	4-methyl pentanal
lutropin	–	LH, luteinizing hormone
oestradiol	–	1,3,5(10)-oestratriene-3,17β-diol
pregnenolone	–	5-pregnene-3β-ol-20-one
testosterone	–	4-androstene-17β-ol-3-one

Chapter 1

INTRODUCTION AND SCOPE OF THIS THESIS

1.1. General Introduction

The testis in man and in general in mammals has two very important functions, i.e. the production of spermatozoa, necessary for sexual reproduction, and the production of male steroid hormones, the androgens, necessary for the development and maintenance of spermatogenesis and primary and secundary sex characteristics.

The production of spermatozoa occurs in a specialized compartment within the testis, viz. the seminiferous tubule (Fig. 1.1). Starting from primordial germ cells, spermatozoa are formed through the intermediate formation of spermatogonia, spermatocytes and spermatids. All the spermatogenic cell types are surrounded by the Sertoli cell which is present in the outer region of the seminiferous tubule. The Sertoli cell more or less guides the whole process of spermatogenesis and is under hormonal regulation of follitropin and androgens. Hypophysectomy, which results in deprivation of hormones, causes drastic disturbances in spermatogenesis, i.e. a large reduction in number and different types of spermatogenic cells.

The production of steroid hormones occurs in the Leydig cells. The Leydig cells are present in the interstitial tissue, the tissue surrounding the seminiferous tubules (see: Fig. 1.1) comprising besides Leydig cells, macrophages, fibroblasts, blood and lymph vessels and nerve endings.

Steroid production by the Leydig cell of e.g. testosterone is influenced by several factors. Lutropin and prolactin (both protein hormones originating from the pituitary) are involved in increased steroid production and formation of lutropin receptors (Purvis et al., 1979). Oestradiol, made from testosterone either peripherally, by Sertoli cells or by Leydig cells (for a review, see: Van der Molen et al., 1981; see also: Rommerts et al., 1982a), impairs steroid production in two ways: 1. either by reducing the synthesis of lutropin in the pituitary with ensueing reduction in blood levels of lutropin (Tcholakian et al., 1978; Chowdhury et al., 1980); 2. or via a local effect in the testis by reducing the activity of Leydig cell enzymes involved in production of testosterone (Dufau et al., 1979; Brinkmann et al., 1980; Nozu et al., 1981). Recent results suggest that Sertoli cells can influence steroidogenesis in Leydig

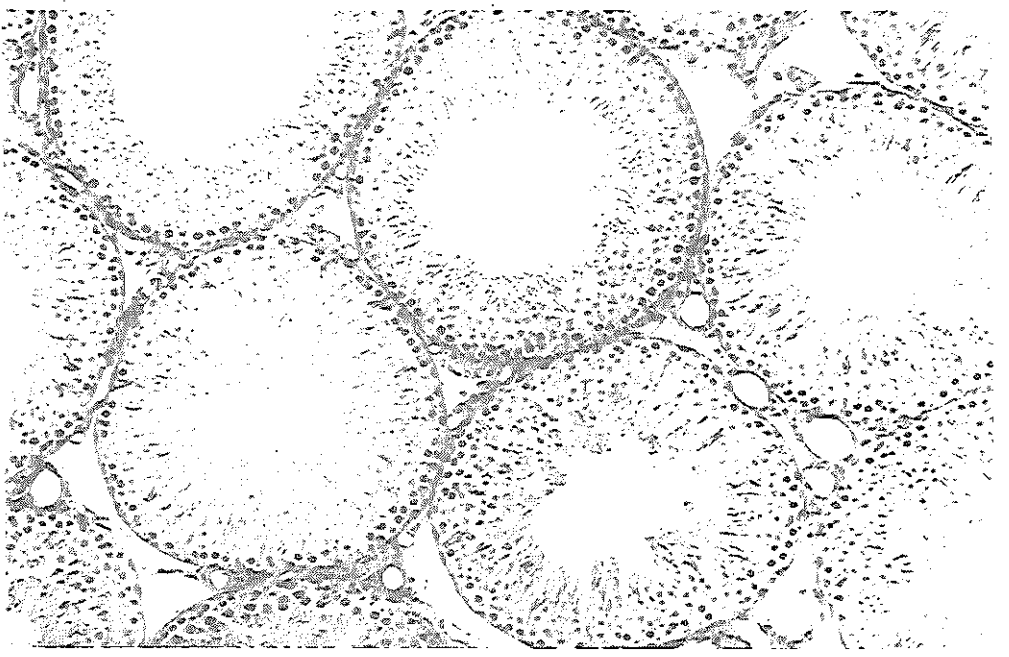


Fig. 1.1. Histology of testicular tissue of an adult rat (fixed with glutaraldehyde and stained with periodic acid Schiff and hematoxylin, x 100). The cylindrical parts represent the seminiferous tubules which contain the germinal cells at different stages of development and Sertoli cells. The interstitial tissue is present in between the seminiferous tubules and comprises Leydig cells, macrophages, fibroblasts, blood and lymph vessels, nerve endings.

cells via a "gonadotropin-releasing hormone-like"-protein (Clayton et al., 1980; Sharpe & Fraser, 1980; Sharpe et al., 1981). The effect of this "gonadotropin-releasing hormone-like"-protein on steroidogenesis can be either inhibitory (Bélanger et al., 1980a,b) or stimulatory (Sharpe & Cooper, 1982).

Steroid production occurs in testis Leydig cells (androgens), ovarian granulosa and theca cells (progestagens and oestrogens), as well as in adrenal fasciculata cells (corticosteroids). Stimulation of steroid production in these various cell types is caused by pituitary hormones, corticotropin, follitropin or lutropin, depending on the steroidogenic cell type. The rate-limiting step in steroid production, stimulated by hormones is the conversion of cholesterol to pregnenolone by the mitochondrial cholesterol side-chain cleavage "enzyme" (for a more detailed description, see: Chapter 2).

1.2. Scope of this thesis

In principle, the work described in this thesis has been concerned with the elucidation of the mechanism of hormonal activation of testicular Leydig cells by lutropin. It has been shown that specific receptors for lutropin are present in the plasma membrane of the Leydig cell (De Kretser et al., 1971a,b). Binding of lutropin to these receptors results in activation of adenylate cyclase to produce cyclic AMP (Cooke et al., 1976). Increased amounts of cyclic AMP can activate cyclic AMP-dependent protein kinase, which has been shown for rat Leydig cells (Cooke et al., 1976) and human adrenal cells (Saez et al., 1978), according to the following equation (Lincoln & Corbin, 1978; Weber & Hilz, 1979):

$$R_2C_2 + 4 \text{ cyclic AMP} \leftrightharpoons R_2\text{-cyclic AMP}_4 + 2C$$

where R_2C_2 is the dimeric, inactive holoenzyme, R is the specific cyclic AMP-binding protein, and C the active catalytic subunit of the kinase. The action of lutropin can be mimicked by dibutyryl cyclic AMP (for a review, see: Marsh, 1976), and it is possible therefore that stimulation of steroid production by lutropin involves phosphorylation of key enzymes concerned in regulation of steroid production. In fact, many cellular responses to hormonal regulation are known to involve phosphorylation of proteins (for a review, see: Lincoln & Corbin, 1978). Initial experiments performed with Leydig cells isolated from adult rat testes demonstrated that lutropin induced phosphorylation of specific proteins in combination with an increased testosterone production (Cooke et al., 1977). Hence, it was decided to investigate the possible role of lutropin-dependent phosphoproteins in the regulation of testicular steroid production. Stimulation of steroid production by lutropin may involve phosphorylation of the cholesterol side-chain cleavage enzyme itself, or, indirectly, the intermediate action of specific phosphoproteins. A schematic representation of the lutropin-dependent formation of phosphoproteins is shown in Fig. 1.2. The indicated effect of lutropin-dependent phosphoproteins on cholesterol availability is discussed in Chapter 2 (microfilaments) and Chapter 5 (specific protein synthesis).

Experiments described in this thesis have been performed with rat tumour Leydig cells because of the almost pure Leydig cell preparations which can be isolated and the ease of isolation of a large number of (tumour) Leydig cells from individual rats. The use of tumour Leydig cells instead of normal testis Leydig cells was justified in view of the similar responses to lutropin with respect to binding of lutropin to its receptor,

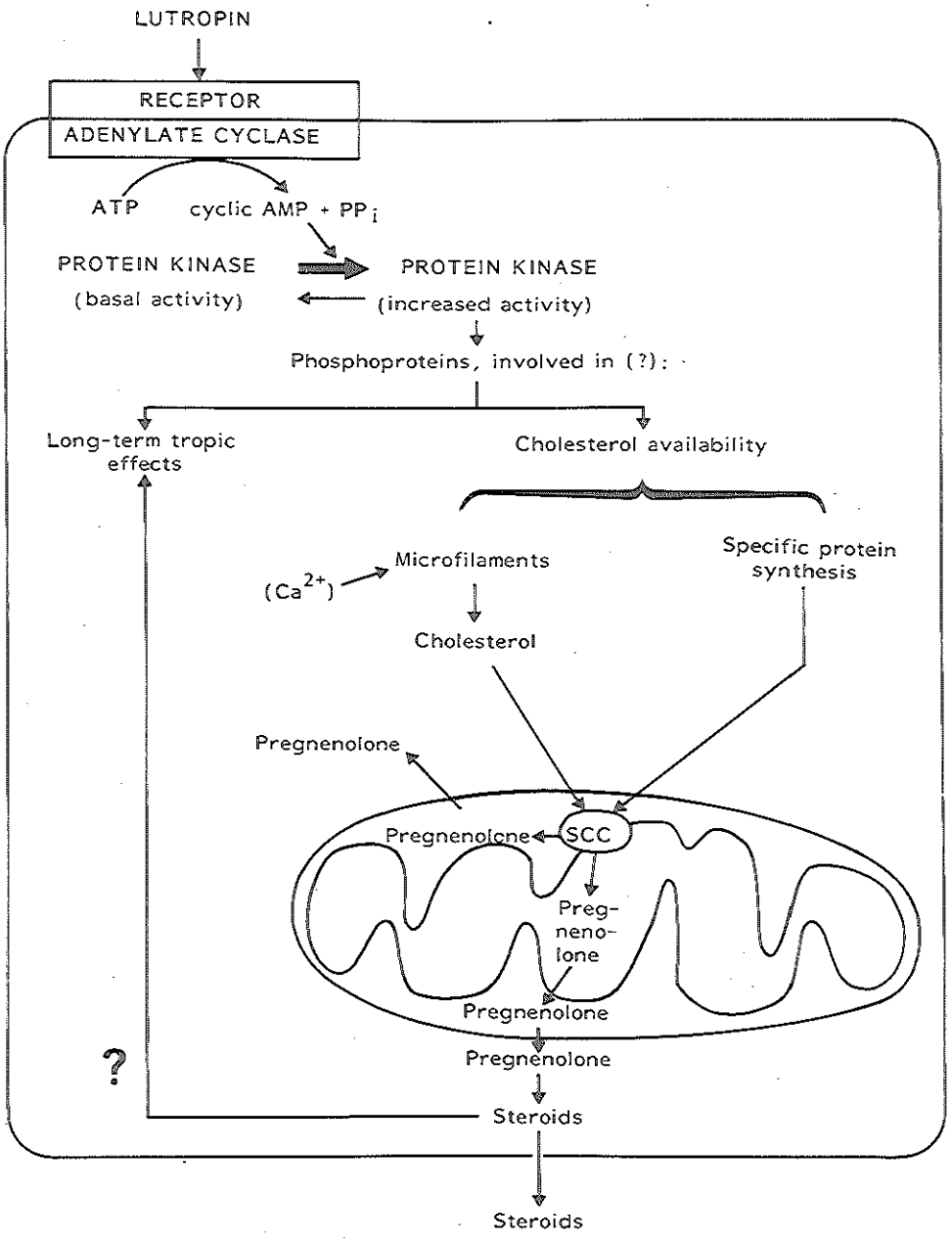


Fig. 1.2. Possible mechanism of lutropin action on (tumour) Leydig cells to increase steroid production. Pregnenolone made from cholesterol may take 2 different routes, as indicated, to get into the cytosol (for explanation, see: section 1.2).

of proteins and increased steroid production (Cooke et al., 1979a). Experiments were designed to study: a. the kinetics of increased pregnenolone production and increased phosphorylation of specific proteins after addition of lutropin; b. the subcellular localization of the lutropin-dependent phosphoproteins with particular reference to mitochondria; c. effects of inhibitors of protein synthesis or of microfilament formation on lutropin-dependent pregnenolone production and phosphorylation of proteins.

On the basis of the results obtained (cf. Chapter 4) possible roles for lutropin-dependent phosphoproteins are discussed in Chapter 5 in combination with a discussion of literature data pertaining to hormonal regulation of steroidogenesis. A hypothesis on the regulation of steroid production is proposed in section 5.4.

Chapter 2

MOLECULAR ASPECTS OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME AND REGULATION OF ITS ACTIVITY BY LUTROPIN

2.1. Introduction

The rate-limiting step of steroid production in steroidogenic cells is the conversion of cholesterol into pregnenolone. Cholesterol used in steroid production is mainly derived from uptake by steroidogenic cells of cholesterol present in high-density lipoproteins (rat), rather than from de-novo synthesis from acetate (Anderson & Dietschy, 1978; Chen et al., 1980; McNamara et al., 1981). Cholesterol in Leydig cells is present almost completely as free (unesterified) cholesterol (see: Van der Molen & Rommerts, 1981). The product of cholesterol side-chain cleavage, pregnenolone, leaks out of the mitochondrion (Shears & Boyd, 1982) and is subsequently transformed to several other steroids, such as progesterone, dehydroepiandrosterone, testosterone, oestradiol (Van der Molen & Rommerts, 1981; Fig. 2.1.). The present knowledge about the cholesterol side-chain cleavage enzyme complex has been reviewed recently by Simpson (1979) and Kimura (1981). This chapter will deal more specifically with the action of the cholesterol side-chain cleavage enzyme complex in some detail to indicate the possible ways in which lutropin-dependent phosphoproteins may control its activity.

2.2. The cholesterol side-chain cleavage enzyme

The conversion of cholesterol into pregnenolone, the actual cleavage of 6 carbon atoms from the cholesterol side-chain, is performed by a complex of 3 different proteins, collectively named the "cholesterol side-chain cleavage enzyme-complex". Purification of the "enzyme" has shown that it consists of:

- a cytochrome P-450 type hemoprotein;
- an NADPH-cytochrome P-450-reductase comprising an FAD-containing flavoprotein and a non-heme iron-sulphur protein (Simpson & Boyd, 1967; Bryson & Sweat, 1968).

The latter two enzyme proteins of the cholesterol side-chain cleavage enzyme have received the following names depending on their origin: adrenodoxin reductase/testodoxin reductase (the FAD-containing flavoprotein); adenodoxin/testodoxin (the non-héme iron-sulphur protein).

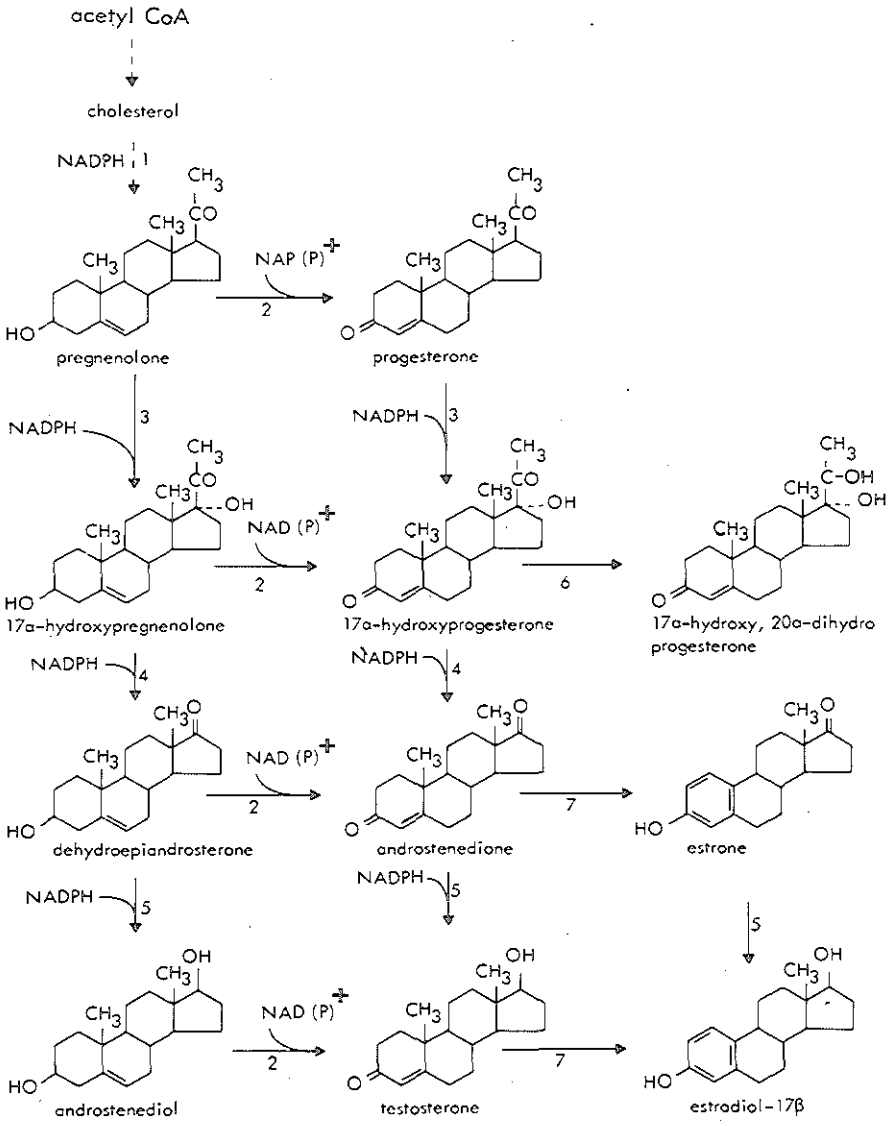


Fig. 2.1. Pathways involved in biosynthesis of testicular steroids from cholesterol: 1. cholesterol side-chain cleavage "enzyme"; 2. 3β-hydroxysteroid dehydrogenase; 3. 17α-hydroxylase; 4. steroid-C_{17-20}-lyase; 5. 17β-hydroxysteroid dehydrogenase; 6. 20α-hydroxysteroid dehydrogenase; 7. aromatizing enzyme complex. The Figure is reproduced by courtesy of Drs. Van der Molen & Rommerts (1981) with minor modifications.

Side-chain cleavage of cholesterol involves two consecutive hydroxylations of carbon atoms 22 and 20, in this order (Alsema et al., 1980; Larroque et al., 1981). Cleavage of the carbon-carbon bond between the positions 20 and 22 of (20R, 22R)20,22-dihydroxycholesterol will finally result in formation of pregnenolone and isocaproaldehyde (see e.g. Larroque et al., 1981). A schematic representation of the cholesterol side-chain cleavage reactions is shown in Fig. 2.2. Reducing equivalents required for the hydroxylations are supplied by NADPH and are transferred to the cytochrome P-450 via adrenodoxin reductase and adrenodoxin (Fig. 2.3). This transfer appears to involve some kind of shuttle mechanism (Kido & Kimura, 1979; Lambeth et al., 1979; Hanugoklu & Jefcoate, 1980). All three components of the cholesterol side-chain cleavage enzyme are present at the inner-side of the inner-mitochondrial membrane. The cytochrome P-450 is embedded in the membrane, adrenodoxin is present at the matrix side of the membrane, whereas adrenodoxin reductase is partially buried in the membrane (Kido & Kimura, 1979), but is also present in the mitochondrial matrix (Lambeth et al., 1980). Adrenodoxin is thought to shuttle reducing equivalents from its reductase to the cytochrome P-450 (Fig. 2.4.).

Accumulation of cholesterol in mitochondria does not lead necessarily to increased steroid production as shown by Farese & Prudente (1978b). In this respect one may consider a metabolic difference between steroidogenic and non-steroidogenic cholesterol present in mitochondria. The presence of cholesterol at the matrix side of the mitochondrion required for binding of cholesterol to the cytochrome P-450. The solubility of unesterified cholesterol in water is very low (Haberland & Reynolds, 1973). In steroidogenic mitochondria, cholesterol is present in the inner- and outer-mitochondrial membranes (Kimura, 1981). The amount of cholesterol present in the inner-half of the inner-mitochondrial membrane could be considered as a steroidogenic cholesterol pool, whereas a non-steroidogenic cholesterol pool could be the residual amount of cholesterol present in the outer-mitochondrial membrane and in the outer-half of the inner-mitochondrial membrane (cf. Mason et al., 1978; Kimura, 1981). Depletion of the steroidogenic cholesterol pool (by side-chain cleavage) is counteracted by redistribution of cholesterol over the mitochondrial membranes. This redistribution might involve inter-membrane transport and intra-membrane "flip-flop" of cholesterol (cf. Kimura, 1981; see also: section 5.4).

The rate-limiting aspect of cholesterol side-chain cleavage appears to be a limited availability of cholesterol for the cytochrome P-450 (e.g. Farese & Prudente, 1978a; Toaff et al., 1979). The cholesterol binding site of the cytochrome P-450 is buried in the hydrophobic phospholipid bilayer (Seybert et al., 1979) and binding of cholesterol is a very rapid process.

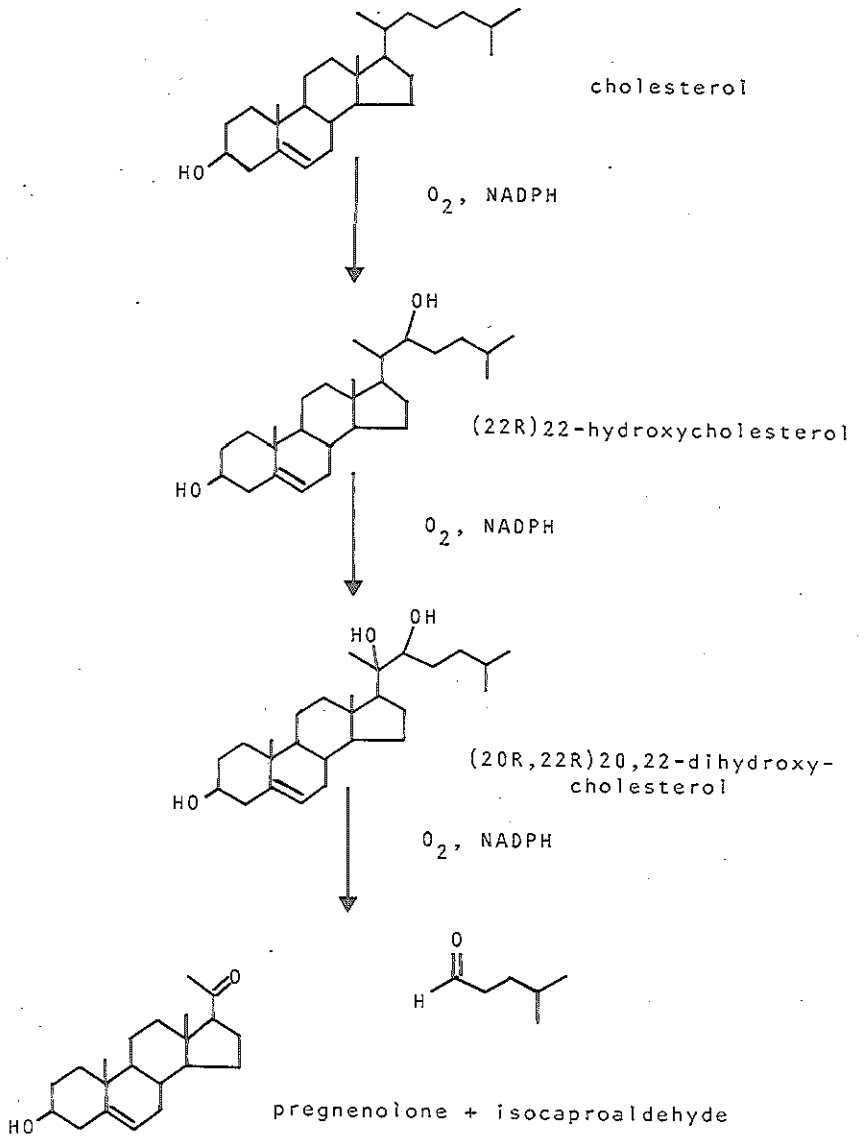


Fig. 2.2. Cholesterol side-chain cleavage.

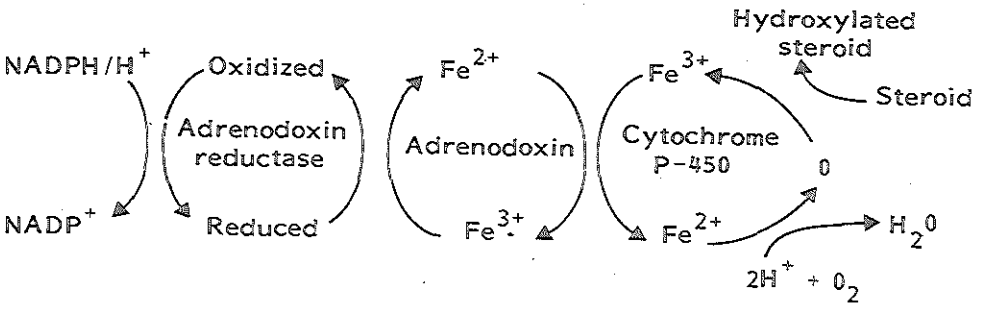


Fig. 2.3. Mechanism of action of the cholesterol side-chain cleavage "enzyme".

Stimulation of steroid production is supposed to involve an increase in the availability of cholesterol for the cytochrome P-450. Several theoretical possibilities can be considered which could result in increased amounts of cholesterol available for side-chain cleavage (Fig. 2.5):

1. uptake of extracellular cholesterol, which is supposed to be the major source of cholesterol (Gwynne et al., 1976; Watanuki & Hall, 1979);
2. release of cholesterol from cholesterol esters stored in fat droplets by the action of cholesterol esterase (Trzeciak & Boyd, 1973);
3. transport of cholesterol through the cell to the mitochondrion by means of the cytoskeleton (Crivello & Jefcoate, 1979);
4. increased transport of cholesterol across the mitochondrial membranes to increase the steroidogenic pool of cholesterol. This process may involve the action of a labile or rapidly-turning-over protein (to be discussed in sections 4.5 and 5.3.3) as suggested by e.g. Jefcoate et al. (1974), Farese & Prudente (1978b);
5. some effect on the inner-mitochondrial membrane (or perhaps on the cholesterol side-chain cleavage enzyme) to improve the association of cholesterol with the cytochrome P-450 (e.g. Kido et al., 1979; Hanugoklu & Jefcoate, 1980).

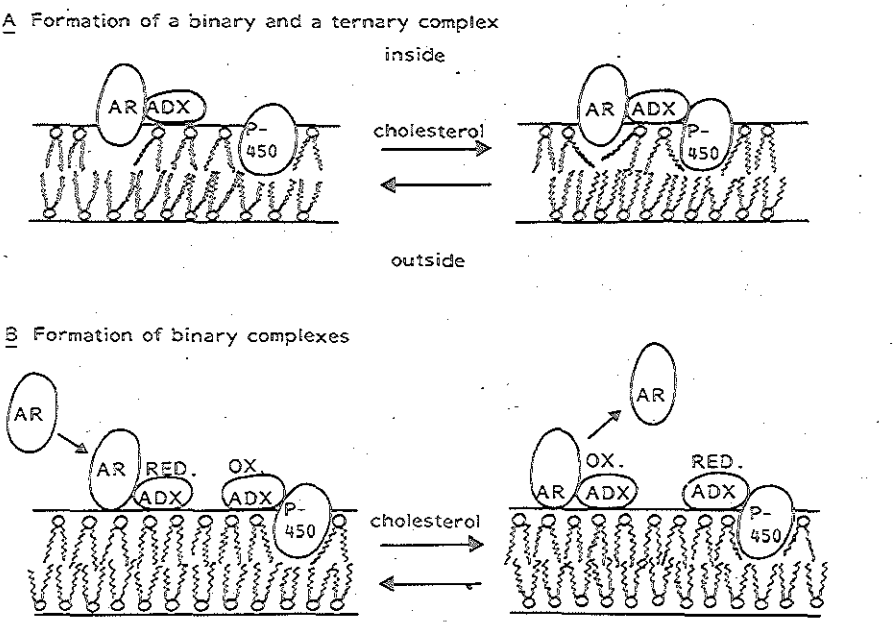


Fig. 2.4. Proposed shuttle mechanism for adrenodoxin in transfer of electrons from adrenodoxin reductase to cytochrome P-450. Abbreviations: AR, adrenodoxin reductase; ADX, adrenodoxin; P-450, cytochrome P-450; OX, oxidized; RED, reduced; inside, matrix side of the mitochondrial membrane; A, Kido & Kimura, 1979; B, Lambeth et al., 1979, Light & Orme-Johnson, 1981.

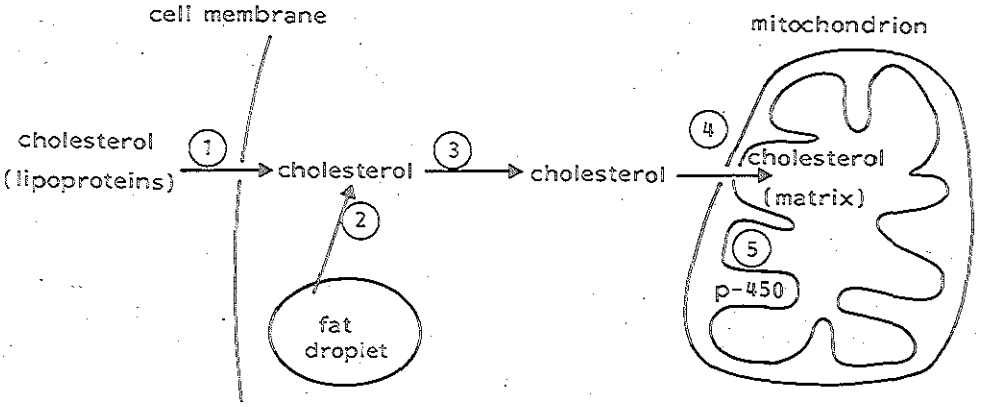


Fig. 2.5. Possible ways to increase the availability of cholesterol for the mitochondrial cholesterol side-chain cleavage enzyme (for explanation, see: section 2.2).

2.3. Availability of cholesterol

In the rat, cellular cholesterol is mainly derived from high-density lipoproteins (cf. section 2.1). This is different from the human situation in which it is mainly derived from low-density lipoproteins (Carr et al., 1980; Winkel et al., 1980). These lipoproteins are probably synthesized as the consequence of the action of lipoprotein lipase (present on the outside of e.g. blood vessel endothelial cells) on very low-density lipoproteins and chylomicrons circulating in the blood (Tall & Small, 1978; Assman & Schriewer, 1980). Uptake of lipoproteins by steroidogenic cells is initiated after binding of the lipoprotein to its receptor (e.g. Chen et al., 1980). Uptake of high-density lipoproteins by rat ovaries could be stimulated by lutropin (Strauss III et al., 1982) and uptake of low-density lipoproteins by mouse tumour adrenal cells could be stimulated by corticotropin (Hall & Nakamura, 1979). Intracellular transport of cholesterol to the mitochondria is also stimulated by hormones as indicated by the increased cholesterol content of mitochondria in response to hormonal stimulation of cells (Farese & Prudente, 1978b). Cellular uptake and intracellular transport phenomena appear to involve the cytoskeleton (e.g. Crivello & Jefcoate, 1978; Murono et al., 1982). The cytoskeleton is constituted of protein fibers of different diameter present in the cytoplasm and nucleoplasm (Chaly et al., 1977; Henderson & Weber, 1979; De Brabander, 1982). Among the various protein fibers one can discern microfilaments (fibers constituted of contractile proteins, i.e. actin, myosin) and microtubules (fibers constituted of tubulin).

The cytoskeleton enables a very orderly organization and localization of the various components present inside the cell. For example, mitochondria appear to be in close association with microtubules (Heggeness et al., 1978; Aufderheide, 1979, 1980; Pardue et al., 1981; Ball & Singer, 1982) and microfilaments (David-Ferreira & David-Ferreira, 1980; see also: Ball & Singer, 1982). Even the activity of some enzymes may be controlled by the cytoskeleton. For example, microtubules appear to be involved in regulation of activity of adenylate cyclase (Hagmann & Fishman, 1980; Rasenick et al., 1981). Moreover, cyclic AMP-dependent protein kinase has been localized on microtubules (e.g. Browne et al., 1982).

Lutropin- or corticotropin-stimulated steroid production is inhibited by disruption of microfilaments with inhibitors such as cytochalasin B or by cellular uptake of liposomes containing anti-actin (Crivello & Jefcoate, 1978, 1979; Hall et al., 1979a; Silavin et al., 1980). Contradictory results have been obtained with rat adrenal and luteal cells regarding the involvement of microtubules in increased steroid production (e.g. Crivello

& Jefcoate, 1978; Azhar & Menon, 1981). Cholesterol transport to mitochondria has been implicated in the studies mentioned above.

The rat adrenal appears to have a considerable amount of cholesterol present in an esterified form (Davis & Garren, 1966; Trzeciak & Boyd, 1973). Hydrolysis of the cholesterol esters can be stimulated by corticotropin (Vahouny et al., 1978). The release of cholesterol from intracellular esterified stores is under the influence of cholesterol ester hydrolase, a protein present in the cytosol and consisting of four subunits of approximately 41000 Da (Beckett & Boyd, 1977).

In what way then could it be possible for lutropin to increase the availability of cholesterol for the cholesterol side-chain cleavage enzyme via phosphorylation of proteins? Apart from an effect on specific protein synthesis (Fig. 1.2; see: Discussion in Chapter 5), one may consider effects on the cytoskeleton, or on the cholesterol ester hydrolase. Regarding the latter, it has been described that stimulation of the activity of cholesterol ester hydrolase by corticotropin appears to involve phosphorylation of the enzyme (Beckett & Boyd, 1977; for a review, see: Boyd & Gorban, 1980). In contrast to adrenal cells, cholesterol in Leydig cells is present almost entirely in an unesterified form, which may circumvent the need of activation of cholesterol ester hydrolase in Leydig cells by lutropin.

Phosphorylated proteins are probably involved in regulation of the cytoskeleton. Most of the present literature data refer to phosphorylation of myosin light-chain, a protein of 20000 Da present in microfilaments of muscle and non-muscle cells (for reviews: Adelstein & Eisenberg, 1980; Stull, 1980). Contraction of microfilaments correlated with phosphorylation of myosin light-chain (Janis et al., 1980; Somlyo et al., 1982). Phosphorylation of myosin light-chain is performed by myosin light-chain kinase. The activity of myosin light-chain kinase can be stimulated in a Ca^{2+}/calmodulin-dependent process (cf. Bhalla et al., 1982). However, cyclic AMP-dependent phosphorylation of the kinase results in decreased activity of myosin light-chain kinase (Adelstein et al., 1978; Bhalla et al., 1982), and the degree of phosphorylation of myosin light-chain will decline concomitant with the decline in activity of its kinase (see: Adelstein et al., 1978). Perhaps, lutropin action in tumour Leydig cells may involve a similar phosphorylation of microfilament proteins. Whether this kind of altered phosphorylation of microfilament proteins might apply to stimulation of Leydig cells by lutropin will be further discussed in section 4.5.

2.4. Regulation of mitochondrial enzyme activities

Stimulation of Leydig cells with lutropin results in activation of the mitochondrial cholesterol side-chain cleavage enzyme which results in increased steroid production. In one way or another some molecular signal should be produced after binding of lutropin to its receptor in the cell membrane to bring about stimulation of the cholesterol side-chain cleavage enzyme.

Hormones such as glucagon, α-adrenergic hormones or vasopressin may control activity of liver enzymes involved in glycolysis, fatty acid synthesis, the citric acid cycle. This appears to involve an increased cytosol calcium concentration by stimulation of Ca^{2+}-release from mitochondria (for a recent review, see: Williamson et al., 1981). The effect of insulin on these liver enzymes is quite the opposite of what is observed in the presence of e.g. glucagon. The exact relationship of insulin to intracellular calcium homeostasis is still rather confused (cf. Williamson et al., 1981). Perhaps the effects of insulin on cellular metabolism are mediated by small protein factors released from the plasma membrane as shown for insulin activation of pyruvate dehydrogenase (Kiechle et al., 1981; Seals & Czech, 1981).

In relation to steroid production, the presence of stimulatory factors in cytosol and post-mitochondrial supernatants isolated from testes or adrenal cells treated with hormone in-vivo and in-vitro respectively, has been documented (Bakker et al., 1978; Neher et al., 1982). Apart from protein factors, including polylysine (Mason et al., 1978; Kido & Kimura, 1981), polyphospholipids may be involved in regulation of mitochondrial cholesterol side-chain cleavage activity (Farese & Sabir, 1979; Farese et al., 1980a; for discussion, see: Chapter 5).

2.5. Conclusions

Increased steroid production in the presence of hormone appears to result from increased availability of cholesterol for the mitochondrial cholesterol side-chain cleavage enzyme. Availability of cholesterol may be increased by processes related to microfilaments and protein synthesis.The way to actually increase the availability of cholesterol may involve: phosphorylated proteins, alterations in the intracellular Ca^{2+}-homeostasis, polyphospholipids, polylysine, protein factor(s) or soluble factor(s) of unknown character.

Chapter 3

MATERIALS AND METHODS

3.1. Introduction

Regulation of steroidogenesis by lutropin was studied in Leydig cells isolated from Leydig cell tumours by collagenase dispersion of tumour tissue fragments (Fig. 3.1). The properties of the isolated cells have been described (Cooke et al., 1979a). Tumour Leydig cells were incubated generally at 32°C (in some experiments at 37°C) under a humidified atmosphere of 5% CO_2 in air, to study the effects of lutropin on synthesis of steroids (pregnenolone) and phosphorylation of proteins. Subcellular fractions were isolated to investigate the subcellular localization of the effects elicited by lutropin. An attempt was made to characterize further the lutropin-dependent pregnenolone production and phosphorylation of proteins by using inhibitors of different cellular processes. Some experiments were performed with Leydig cells from immature and mature rat testes (cf. Appendix Paper II).

In general, stimulation of 1-2 x 10^6 Leydig cells was performed with ovine lutropin (tumour Leydig cells: 1000 ng/ml; immature and mature Leydig cells: 100 ng/ml) and 1-methyl-3-isobutylxanthine (0.25 mM), a phosphodiesterase inhibitor. Addition of 1-methyl-3-isobutylxanthine to Leydig cells isolated from mature rat testes significantly increased

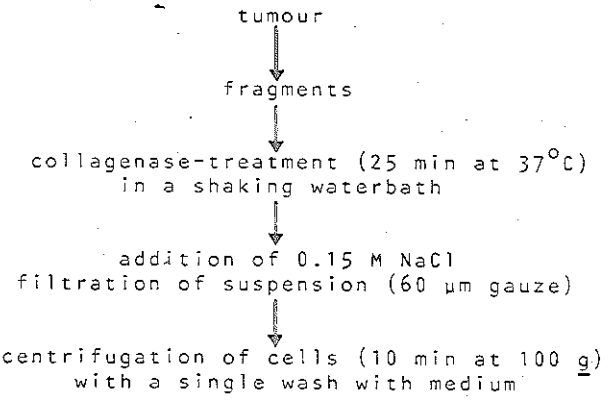


Fig. 3.1. Scheme for isolation of tumour Leydig cells.

synthesis of the lutropin-induced protein in the presence of a submaximal dose of lutropin, although no effect of this inhibitor could be observed with maximally stimulating amounts of lutropin (Janszen et al., 1978a). Addition of 1-methyl-3-isobutylxanthine slightly stimulated control and lutropin-stimulated pregnenolone production. Positive (stimulatory) effects of 1-methyl-3-isobutylxanthine on lutropin-dependent pregnenolone production and phosphorylation of proteins were taken into consideration during its continued application (with lutropin) in the experiments.

3.2. Estimation of pregnenolone production

Estimation of steroid production was directed at the assay of the amount of pregnenolone produced, since pregnenolone is the first steroid molecule synthesized from cholesterol by the mitochondrial cholesterol side-chain cleavage enzyme. To estimate the amount of pregnenolone produced in steroidogenic cells it is essential to block the activity of pregnenolone metabolizing enzymes. In tumour Leydig cells no pregnenolone production could be demonstrated in the absence of inhibitors of pregnenolone metabolism. Inhibitors used were cyanoketone (5 μM; inhibitor of Δ^5, 3β-hydroxysteroid dehydrogenase) and SU 10603 (19 μM; inhibitor of 17α-steroid hydroxylase) (for details on enzymatic conversions inhibited, see: Fig. 2.1). A 50 times concentrated solution of these inhibitors was prepared in ethanol/medium (1:9, by vol.). Final concentration of ethanol during incubation of cells was approximately 0.2%. The presence of 2% ethanol during incubation of cells gave rise to a decrease in the amount of pregnenolone assayed of approximately 25%. The degree of inhibition of pregnenolone metabolism in the presence of the inhibitors was approximately 50% (cf. Cooke et al., 1979a).

In experiments on the kinetics of increased (with lutropin) and decreased (with cycloheximide, see: section 4.5) pregnenolone production, the amount of pregnenolone present at the end of the various incubation periods used, should be determined as accurate as possible. To this end, cells including medium were extracted with ethyl acetate. In all other experiments the medium only was extracted, since the amount of pregnenolone retained by the cells was approximately 10% (Rommerts et al., 1982b). Pregnenolone production was expressed relative to the number of cells (i.e. per 10^6 cells) or to the amount of protein.

Various inhibitors have been tested for their possible effects on lutropin-dependent pregnenolone production and phosphorylation of proteins. To evaluate possible damage of the cholesterol side-chain cleavage enzyme,

pregnenolone production in the presence of 25-hydroxycholesterol was determined (see: section 4.3; Appendix Paper III). Some inhibitors adversely affected pregnenolone production in the presence of 25-hydroxycholesterol, which might indicate a questionable specificity of the inhibitor used. Cycloheximide (89 μM), for example, did not inhibit pregnenolone production in the presence of 25-hydroxycholesterol (32 μM), indicating that cycloheximide does not impair the cholesterol side-chain cleavage enzyme per sé.

3.3. Phosphorylation of proteins

Tumour Leydig cells were incubated with radioactive phosphate to enable detection of phosphoproteins. Labelling of proteins was performed by incubation of cells in Krebs Ringer buffer devoid of phosphate (cf. Appendix Paper I). Leydig cells were incubated with ^{32}P-phosphate (carrier-free; 75-200 μCi/ml) for 60 min to approximate an intracellular steady state concentration of radioactive phosphate. Incubations with ^{32}P-phosphate for 30 min or less, clearly showed an effect of lutropin/1-methyl-3-isobutylxanthine on uptake of the ^{32}P-phosphate. The latter interfered with a proper appraisal of the effects of lutropin on phosphorylation of proteins. Phosphorylation of proteins in Leydig cells was stopped by the addition of a cold (0°C) medium containing fluoride and phosphate (inhibition of phosphatases). This medium was made iso-osmotic in order not to impair subsequent subcellular fractionation (see: next section). Phosphorylated proteins were isolated via: lysis of cells with sodium dodecyl sulphate (SDS), precipitation of (phosphorylated) proteins with acetone, washing of the phosphorylated proteins to remove radioactive phosphate and phospholipids/lipoproteins. Finally, proteins were dissolved in an SDS-containing sample medium enabling SDS-polyacrylamide gel electrophoresis of phosphorylated proteins. For quantitative evaluation of the effects of lutropin on protein phosphorylation, see below (section 3.5).

3.4. Isolation and incubation of subcellular fractions

Subcellular fractions isolated from tumour Leydig cells were: nuclei, mitochondria and post-mitochondrial supernatant (Fig. 3.2). The post-mitochondrial supernatant was further separated in microsomes and cytosol. Approximately 20-40 x 10^6 tumour Leydig cells were used for preparation of subcellular fractions. Nuclei, mitochondria and post-mitochondrial supernatant fractions isolated were characterized on the basis of the

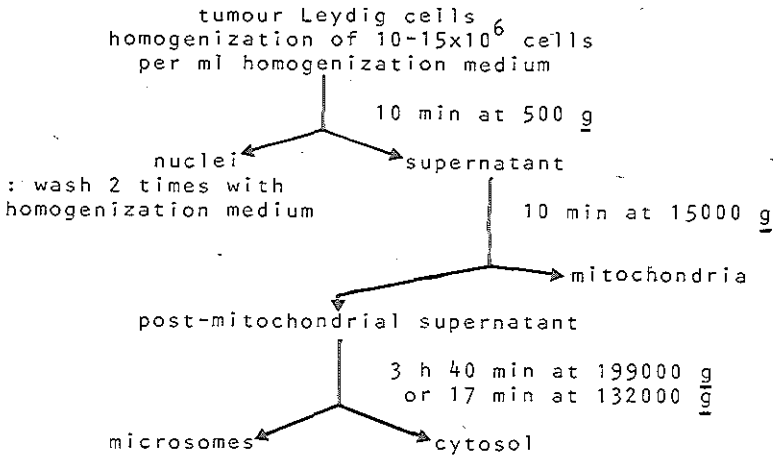


Fig. 3.2. Scheme for isolation of subcellular fractions from tumour Leydig cells.

distribution of marker enzymes.

Subcellular fractionation of tumour Leydig cells was performed after disruption of cells with a Dounce glass homogenizer (cf. Appendix Paper I), with a motor-driven teflon-glass Potter tube (3 x 20 sec at 1100 cycles per min), with sonication (Gellerfors & Nelson, 1979) or by using nitrogen pressure homogenization (Hunter & Commerford, 1961). The Dounce homogenizer gave the best results for homogenization of cells as reflected in an adequate distribution of marker enzymes.

Tumour Leydig cells were pretreated with or without lutropin/1-methyl-3-isobutylxanthine prior to subcellular fractionation. For pregnenolone production, subcellular fractions isolated were incubated in a sucrose medium fortified with an NADPH-generating mixture (cf. Van der Vusse et al., 1974), but lacking calcium to avoid damage of mitochondria inflicted by calcium (see: section 5.3.2). Because of the mitochondrial localization of the cholesterol side-chain cleavage enzyme, special care was bestowed on isolation of mitochondria. The intactness of the mitochondrial membranes was tested with the NADPH-generating mixture in incubations for pregnenolone production. Pregnenolone production in freshly isolated cells was not increased by addition of this NADPH-generating mixture, indicating the presence of intact cell membranes. When membranes of isolated mitochondria were disrupted by freezing or sonication, pregnenolone production was completely dependent on the presence of the NADPH-generating mixture. To estimate the relative intactness of the isolated mitochondria, pregnenolone production was determined in the absence or presence of the

NADPH-generating mixture. Pregnenolone production in the absence of the NADPH-generating mixture was 45% ± 15 (mean ± S.D.; n = 6) of the pregnenolone production in the presence of the NADPH-generating mixture.

Phosphorylated proteins in subcellular fractions were isolated after prelabelling of tumour Leydig cells with ^{32}P-phosphate. To study possible dephosphorylation which might occur during isolation of subcellular fractions, aliquots of the cellular homogenate were kept on ice and phosphorylated proteins were isolated after trichloroacetic acid precipitation of proteins at different times. Isolation of the post-mitochondrial supernatant (which took about one hour) did not appear to affect significantly phosphorylation of lutropin-dependent phosphoproteins. However, dephosphorylation of lutropin-dependent phosphorylation was conspicuous after isolation of microsomes and cytosol (which took another 4 hours). Isolation of microsomes and cytosol was also performed very rapidly using the Airfuge (17 min) (Fig. 3.2; Appendix Paper IV).

3.5. Separation and estimation of phosphorylated proteins

Phosphorylated proteins were separated using SDS-polyacrylamide gel electrophoresis with SDS/8-15% polyacrylamide slab gels. After fixation, staining and drying of slab gels, autoradiography was performed. The autoradiogrammes were scanned in order to obtain densitogrammes.

Estimation of the ^{32}P-incorporation into lutropin-dependent phosphoproteins at the cellular level was performed with densitograms, by comparison of peak heights of lutropin-dependent phosphoproteins with the peak height of a lutropin-independent phosphoprotein, resulting in a peak height ratio. A relative specific activity for lutropin-dependent phosphoproteins in subcellular fractions (based on peak height) was calculated. Subcellular localization of lutropin-dependent phosphoproteins was based on comparison of either relative specific actitivies of lutropin-dependent phosphoproteins and markers (for nuclei, mitochondria, post-mitochondrial supernatant), or peak height patterns in densitograms (for microsomes, cytosol).

Chapter 4

EFFECTS OF LUTROPIN ON TUMOUR LEYDIG CELLS

4.1. Introduction

This chapter deals with results obtained with tumour Leydig cells. The effects of lutropin on pregnenolone production and phosphorylation of proteins were studied to find out whether phosphorylated proteins might be involved in regulation of steroid production. The correlation of the effects of lutropin on pregnenolone production and phosphorylation of proteins was studied either with freshly isolated cells (section 4.2) or with cells incubated for two days (section 4.3). In other experiments the subcellular localization of lutropin-dependent phosphoproteins was determined (section 4.4). Finally, the possible involvement of specific phosphoproteins in protein synthesis and in microfilaments has been considered (section 4.5).

4.2. Kinetic studies of lutropin-dependent pregnenolone production and protein phosporylation (Appendix Paper I)

Incubation of tumour Leydig cells at 32°C with lutropin/1-methyl-3-isobutylxanthine, resulted in increased pregnenolone production (Fig. 4.1) and increased phosphorylation of at least five proteins (Fig. 4.2) within 5 min after addition of lutropin/1-methyl-3-isobutylxanthine. The molecular masses of the lutropin-dependent phosphoproteins were: 17000, 20000, 22000, 24000, 33000, 43000, 57000 and 76000 Da. The 20000 Da protein was dephosphorylated (see: next section) under the influence of lutropin. By comparison of peak height ratios it was concluded that phosphorylation of at least the lutropin-dependent phosphoproteins of 17000, 22000, 24000, 33000 and 57000 Da was significantly increased within 5 min after the addition of lutropin/1-methyl-3-isobutylxanthine. The similar kinetics of increased pregnenolone production and phosphorylation of these specific proteins in the presence of lutropin/1-methyl-3-isobutylxanthine suggested that phosphorylation of these proteins may be involved in lutropin-dependent steroid production.

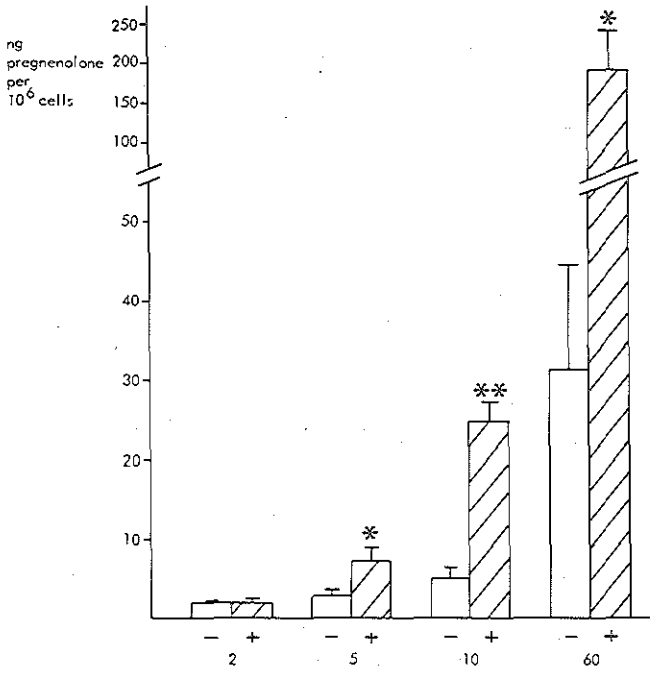


Fig. 4.1. Effect of incubation time on pregnenolone production in tumour Leydig cells in the absence (-) or presence (+) of lutropin/1-methyl-3-isobutylxanthine. Tumour Leydig cells were incubated for 60 min, and lutropin/1-methyl-3-isobutylxanthine was added with inhibitors of pregnenolone metabolism at different time intervals before the end of the incubation period (as indicated in min). Results shown are means ± S.D. (n = 3); *, $p < 0.025$; **, $p < 0.005$).

4.3. Lutropin-dependent pregnenolone production and protein phosphorylation in cultured tumour Leydig cells (Appendix Paper III)

The fast response of the phosphorylation of specific proteins in freshly isolated tumour Leydig cells to lutropin indicated that lutropin-dependent phosphoproteins could be essential for increased steroid production. However, this fast response made it impossible to assess in more detail the importance of each individual lutropin-dependent phosphoprotein for increased steroid production. Preliminary results with tumour Leydig cells in culture for two days showed a gradually declining response to lutropin of both pregnenolone production and (de)phosphorylation of proteins. The kinetics of the declining responses were investigated because these kinetics might indicate which lutropin-dependent phosphoprotein correlated best with lutropin-dependent

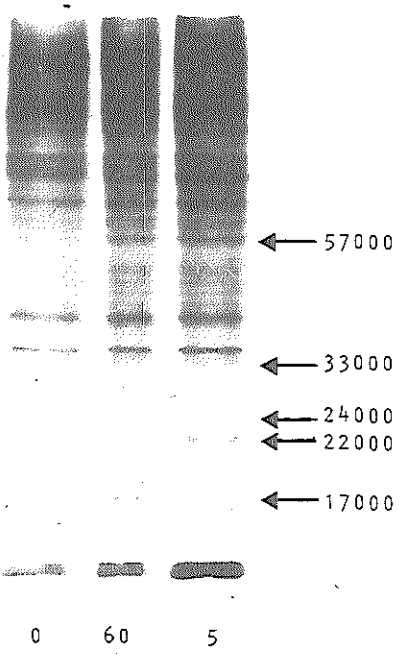


Fig. 4.2. Kinetics of lutropin-dependent phosphorylation of specific proteins in tumour Leydig cells. Tumour Leydig cells were incubated for 60 min with radioactive phosphate, and lutropin/1-methyl-3-isobutylxanthine was added at different time intervals before the end of the incubation period (as indicated in min). The figure shows the autoradiogram obtained after SDS-polyacrylamide gel electrophoresis of the isolated phosphorylated proteins. Arrows indicate lutropin-dependent phosphoproteins and their molecular masses in daltons.

pregnenolone production, i.e. which phosphoprotein could be essential for increased steroid production.

In this series of experiments, incubations were also performed with dibutyryl cyclic AMP and 25-hydroxycholesterol. The response of cells to dibutyryl cyclic AMP might indicate whether formation of endogenous cyclic AMP was impaired. The response of cells to 25-hydroxycholesterol was used to determine changes in the activity of the cholesterol side-chain cleavage enzyme (Mason & Robidoux, 1978; Alsema et al., 1980; Toaff et al., 1982). During culture of cells for two days at 32°C, the responsiveness was tested in incubations for 60 min in the presence of lutropin/1-methyl-3-isobutylxanthine. It was found that pregnenolone production as well as (de)phosphorylation of proteins was declining, with no effect on lutropin-independent protein phosphorylation. Similar results were obtained with dibutyryl cyclic AMP, which suggested that during culture some defect

occurred in the regulation of steroidogenesis after formation of cyclic AMP. Pregnenolone production in the presence of 25-hydroxycholesterol also declined during culture. However, this decline of pregnenolone production was significantly less than the decline of pregnenolone production during incubations in the presence of lutropin/1-methyl-3-isobutylxanthine or dibutyryl cyclic AMP. These results of similar declines of pregnenolone production and of (de)phosphorylation of proteins did not enable assignment of a specific phosphoprotein as a possible cause for the decreased pregnenolone production. In fact, the similar declines indicated that a common factor was involved. Decreased phosphorylation of the lutropin-dependent phosphoprotein of 57000 Da (the regulatory subunit of the type II cyclic AMP-dependent protein kinase; Cooke et al., 1979d) suggested that the activity of cyclic AMP-dependent protein kinase had changed.

Estimation of the activity of cyclic AMP-dependent protein kinase showed a decline in kinase activity of cells maintained at 32°C. This decline in kinase activity (on a per cent basis) was comparable to the decline in (de)phosphorylation of lutropin-dependent phosphoproteins and in stimulated pregnenolone production in the presence of lutropin/1-methyl-3-isobutylxanthine. However, during culture, stimulated pregnenolone production had decreased somewhat more (on a per cent basis) than the activity of cyclic AMP-dependent protein kinase and (de)phosphorylation of lutropin-dependent phosphoproteins. The decline in kinase activity closely parallelled the decline in phosphorylation of the lutropin-dependent phosphoprotein of 57000 Da (see above). This may reflect a decrease in the amount of cyclic AMP-dependent protein kinase during culture. Hypophysectomy of tumour bearing rats (at day 7 prior to isolation of tumour Leydig cells) did not influence the steroid response to lutropin/1-methyl-3-isobutylxanthine during incubation of cells). Hence, these results suggest that maintenance of cyclic AMP-dependent protein kinase does not require the continuous presence of lutropin.

The more or less similar declines in (de)phosphorylation of proteins and stimulated pregnenolone production observed with tumour Leydig cells during short-term culture suggested that lutropin-dependent phosphoproteins of 20000, 22000, 24000, 33000 and 57000 Da (and possibly 43000 and 76000 Da; cf. Appendix Paper IV) might be essential for acute regulation of steroid production by lutropin (see also: Appendix Paper I). Lutropin-dependent phosphorylation of the 17000 Da protein decreased faster than (de)phosphorylation of all other lutropin-dependent phosphoproteins. In view of the similar residual activities on day 2 of culture of phosphorylation of the 17000 Da protein (9%) and of lutropin-stimulated pregnenolone production (13%) (cf. Appendix Paper III) in combination with

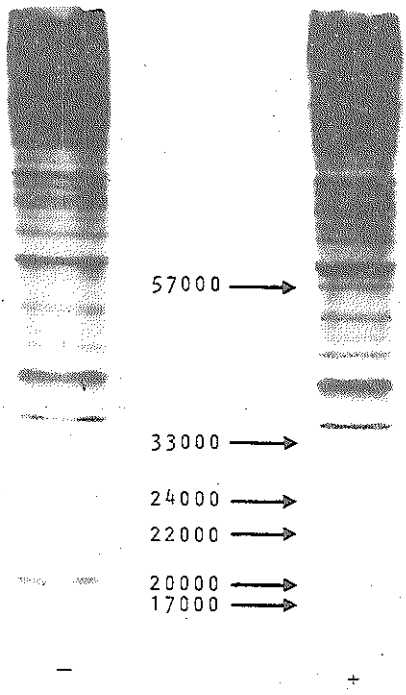


Fig. 4.3. Lutropin-dependent dephosphorylation of a 20000 Da protein. Tumour Leydig cells were incubated for 60 min with radioactive phosphate and without (–) or with (+) lutropin/1-methyl-3-isobutylxanthin (for details, see: legend to Fig. 4.2).

the relatively slow phosphorylation of the nuclear 17000 Da protein (cf. Appendix Paper I), perhaps one might consider this protein to be involved in long-term maintenance of steroid production (see also: section 4.5).

Investigation of tumour Leydig cells in short-term culture showed also the lutropin-dependent dephosphorylation of a protein of 20000 Da (Fig. 4.3). The effect of lutropin/1-methyl-3-isobutylxanthine on dephosphorylation of this 20000 Da protein was also declining during culture similar to the declining phosphorylation of the lutropin-dependent phosphoproteins mentioned above.

Summarizing, it is apparent that during culture of tumour Leydig cells at 32°C a gradual decline in the activity of cyclic AMP-dependent protein kinase occurs. This decline in activity of cyclic AMP-dependent protein kinase may account for the decreased responsiveness of tumour Leydig cells to lutropin with respect to (de)phosphorylation of specific proteins. Moreover, this decline in kinase activity may contribute (i.e. be partly responsible) for the decreased pregnenolone production in the presence of

lutropin/1-methyl-3-isobutylxanthine. Lutropin-dependent phosphoproteins may be essential for acute and long-term regulation of steroid production.

4.4. Subcellular localization of lutropin-dependent phosphoproteins (Appendix Papers I, II and IV) and pregnenolone production by isolated mitochondria (Appendix Paper I)

The rate-limiting step in steroidogenesis is the conversion of cholesterol into pregnenolone by the cholesterol side-chain cleavage enzyme which is present in mitochondria (see: section 2.2). It was of great interest therefore to investigate whether the subcellular localization of the lutropin-dependent phosphoproteins would indicate a possible relationship with the localization and activity of the cholesterol side-chain cleavage enzyme in mitochondria. However, it was found that none of the lutropin-dependent phosphoproteins of 17000, 20000, 22000, 24000, 33000, 43000, 57000 and 76000 Da was present in mitochondria.

The 17000 Da lutropin-dependent phosphoprotein was isolated in the nuclear fraction, the 24000 and 33000 Da phosphoproteins were isolated with the microsomes, while phosphoproteins of 20000, 22000, 43000 and 76000 Da were isolated in the cytosol. The 57000 Da lutropin-dependent phosphoprotein was present in microsomes (approx. 30%) and cytosol (approx. 70%). The presence of lutropin-dependent phosphoproteins in microsomes and cytosol isolated from tumour Leydig cells is shown in Figure 4.4. Further investigation of lutropin-dependent phosphoproteins showed that the 33000 Da microsomal phosphoprotein was present in 40S ribosomal subunits (Appendix Paper II). This lutropin-dependent phosphoprotein of 33000 Da appears similar to the ribosomal protein S6 with regard to its molecular mass, its presence in 40S ribosomal subunits, and its sensitivity towards phosphorylation in the presence of inhibitors of protein synthesis.

Pregnenolone production in mitochondria isolated from non-stimulated tumour Leydig cells or cells previously treated with lutropin/1-methyl-3-isobutylxanthine for 60 min, and frozen prior to incubation for another 30 min with an NADPH-generating mixture (cf. Van der Vusse et al., 1974) was approximately the same (900-1000 ng/mg protein). However, isolation of mitochondria from control and lutropin/1-methyl-3-isobutylxanthine-stimulated cells, without freezing of mitochondria, showed a 2.5-fold increased pregnenolone production in incubation of mitochondria from stimulated cells as compared to mitochondria from control cells (cf. Appendix Paper I).

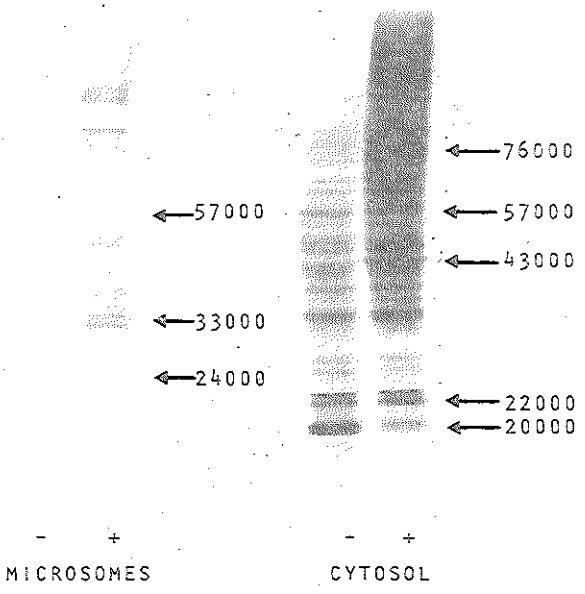


Fig. 4.4. Localization of lutropin-dependent phosphoproteins in microsomes and cytosol isolated from tumour Leydig cells incubated for 60 min with radioactive phosphate and without (-) or with (+) lutropin/1-methyl-3-isobutylxanthine (for details, see: legend to Fig. 4.2).

These results may indicate that control mitochondria contained sufficient cholesterol for the lutropin-dependent pregnenolone production (see also: Jefcoate et al., 1974). Perhaps this amount of cholesterol was constituting the non-steroidogenic pool of cholesterol (cf. section 2.2), not readily accessible for the cholesterol side-chain cleavage enzyme. Freezing of mitochondria might have rendered the non-steroidogenic cholesterol steroidogenic which was used for pregnenolone production in the presence of NADPH.

4.5. Involvement of microfilaments and protein synthesis in lutropin action (Appendix Paper IV)

Several published studies have demonstrated the inhibitory action of inhibitors of microfilament formation and inhibitors of protein synthesis on hormone-induced steroid production (for discussion, see: sections 2.3 and 5.3.3 resp.). It appeared very likely therefore, that hormonal regulation of steroid production might involve activation of microfilaments and synthesis of some protein factor. As a consequence, the availability of cholesterol for the cholesterol side-chain cleavage enzyme might increase as a result of the actions of microfilaments and this protein factor (cf. section 2.2).

The lutropin-dependent phosphoproteins of 20000, 43000 and 76000 Da present in the cytosol isolated from tumour Leydig cells may be related to microfilaments. The molecular masses, the isolation in the cytosol, the phosphorylation of the 76000 Da protein and the dephosphorylation of the 20000 Da protein indicated that the 20000 and 76000 Da lutropin-dependent phosphoproteins are very similar to the microfilament proteins myosin light-chain (the 20000 Da protein) and its kinase (the 76000 Da protein) (cf. Bhalla et al., 1982; section 2.3). In addition, the lutropin-dependent phosphoprotein of 43000 Da, which is also present in the cytosol, and actin have the same molecular mass (e.g. Riddle et al., 1979a). Incubation of tumour Leydig cells with cytochalasin B (50 μM; cf. Murono et al., 1980; Azhar & Menon, 1981) to disrupt microfilaments resulted in a decrease of control and lutropin-dependent pregnenolone production by approximately 60% and 45% respectively, but control and lutropin-dependent phosphorylation of proteins were unaffected (Appendix Paper IV). In general, control steroid production is not influenced by inhibitors of microfilaments or protein synthesis. The fact that control pregnenolone production was inhibited by cytochalasin B may be explained by a slightly stimulated pregnenolone production in tumour Leydig cells, which has been suggested to account also for the inhibitory effect of cycloheximide on control pregnenolone production (cf. Appendix Paper II). Addition of cytochalasin B had no effect on phosphorylation of proteins, which may reflect that activation of cyclic AMP-dependent protein kinase does not involve microfilaments.
These results suggested that microfilaments could be involved in lutropin regulation of steroid production in tumour Leydig cells. From these results it cannot be excluded that activation of microfilaments occurs via (de)phosphorylation of lutropin-dependent phosphoproteins (see also: section 2.3).

Protein synthesis is necessary for hormone-dependent steroid production (for discussion: section 5.3.3). The lutropin-dependent phosphoprotein of 17000 Da was isolated with the nuclear fraction, which suggested a possible role of this phosphoprotein in protein synthesis at the level of DNA-transcription. Addition of actinomycin D (10 μg/ml) to 2 h preincubated cells did not affect lutropin-dependent pregnenolone production within one hour. Therefore, synthesis of (m)RNA and the lutropin-dependent phosphorylation of the nuclear protein of 17000 Da (see: section 4.3) may be of little significance for acute regulation of steroid production.

Still, a possible protein factor appears to be required for hormone-

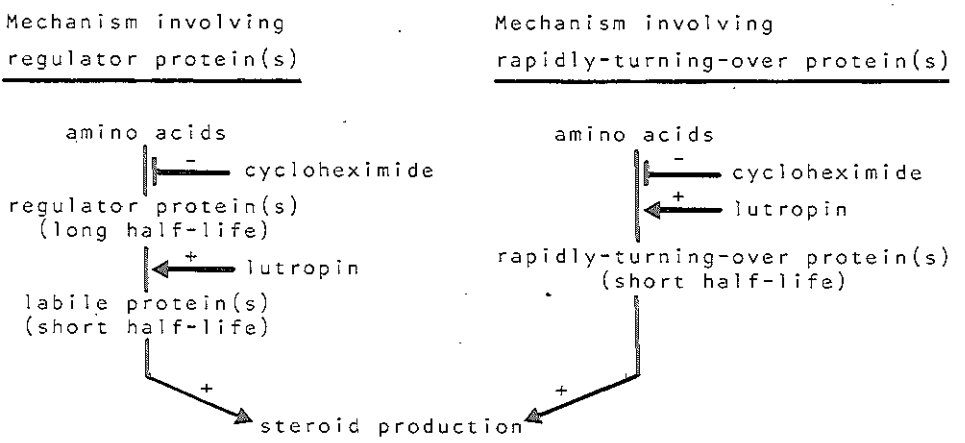


Fig. 4.5. Possible mechanisms for the role of protein synthesis in the regulation of steroid production. −, inhibitory; +, stimulatory (for explanation, see: section 4.5).

dependent steroid production on the basis of the inhibition of hormone-dependent steroid production in the presence of inhibitors of mRNA translation, such as cycloheximide and puromycin. The very rapid inhibition of hormone-dependent steroid production resulted in qualifications of the protein factor in question as a "rapidly-turning-over protein(s)" (Garren et al., 1965) and as a "labile protein(s)" (Cooke et al., 1979c). To account for the synthesis of the protein factor, two models have been proposed. The possible protein factor required for hormone-dependent steroid production either could be present as a regulator protein(s), as proposed by Cooke et al. (1979c), or could be newly synthesized as proposed by Garren et al. (1965).

Cooke et al. (1979c) proposed that lutropin stimulation of steroid production in testis Leydig cells caused transformation of a stable regulator protein(s) into a protein with a short half-life (i.e. into a labile protein(s); Fig. 4.5). The stable regulator protein(s) was believed to be synthesized independently of lutropin action. In this respect one might consider the presence of a certain amount of regulator protein(s) in preincubated cells which, in the presence of cycloheximide, would permit a transient increase of steroid production after addition of lutropin (see: Fig. 4.5). However, in perifusion experiments with immature Leydig cells it was not possible to demonstrate a transient response of steroid production. If a regulator protein(s) exists, it is apparently of minor importance to lutropin-dependent steroid production.

As an alternative, the possibility could be considered that hormone-dependent steroid production depends on a newly synthesized rapidly-

turning-over protein(s) (Garren et al., 1965; Fig. 4.5).

Stimulation of tumour Leydig cells with lutropin/1-methyl-3-isobutylxanthine-involved phosphorylation of a protein similar to ribosomal protein S6 (the 33000 Da phosphoprotein), which did not affect general protein synthesis (Appendix Paper II). Hence, a specific effect of lutropin stimulation of tumour Leydig cells on protein synthesis was investigated by labelling of tumour Leydig cell proteins with radioactive amino acids. However, no experimental evidence for synthesis of a specific protein could be obtained after separation of proteins with SDS-polyacrylamide gel electrophoresis. Ribosomal protein S6 and the lutropin-dependent 24000 Da phosphoprotein are present in microsomes. The molecular mass (24000 Da), the subcellular localization and its being phosphorylated (cf. Van Steeg et al., 1981), made it attractive to consider the possibility that the lutropin-dependent phosphoprotein of 24000 Da might be similar to initiation factor eIF-4E (the mRNA-cap binding protein; cf. Thomas et al., 1981). The combined actions of lutropin-dependent phosphoproteins of 24000 and 33000 Da might result in synthesis of the rapidly-turning-over protein(s). To explain the presence of this putative initiation factor of 24000 Da in microsomes, attachment to ribosomes might be considered, when it is phosphorylated in the presence of lutropin.

In this respect a study on induction of tyrosine aminotransferase by dibutyryl cyclic AMP in rat hepatoma cells is relevant (Snoek et al., 1981). Snoek et al. have shown that induction of tyrosine aminotransferase is caused by a specific effect on synthesis of tyrosine aminotransferase, implying increased initiation of translation of the tyrosine aminotransferase mRNA. To evaluate the increased initiation of translation, Snoek et al. studied the sensitivity of general protein synthesis and synthesis of tyrosine aminotransferase to low concentrations of cycloheximide (see also: Brooks, 1977; Riddle et al., 1979b). Cycloheximide inhibits elongation of translation (Siegel & Sisler, 1965). Inhibition of protein synthesis will occur when the rate of elongation becomes less than the rate of initiation. Inhibition of protein synthesis with a low concentration of cycloheximide results in a certain degree of inhibition of elongation (effect on protein synthesis, see: Appendix Paper IV). An apparently stronger inhibition of synthesis of a specific protein will be observed when with the same low concentration of cycloheximide the rate of initiation of translation is increased, e.g. by hormone treatment (Snoek et al., 1981; Monier & Le Marchand-Brustel, 1982). The identification of a rapidly-turning-over protein(s) has not been described up to now, which makes it impossible to study the effects of low concentrations of cycloheximide on synthesis of the presently elusive rapidly-turning-over

protein(s). Nevertheless, it is possible to study effects of low concentrations of cycloheximide on the alleged activity of the rapidly-turning-over protein(s), i.e. on increased steroid production.

Incubation of tumour Leydig cells with low concentrations of cycloheximide resulted in a significantly higher inhibition of lutropin-stimulated pregnenolone production as compared to control pregnenolone production. However, there was no difference in the inhibition of general protein synthesis in control and lutropin-stimulated cells. Analogous to the results described by Snoek et al. (1981), the significantly higher inhibition of lutropin-dependent pregnenolone production by low concentrations of cycloheximide may suggest that stimulation of steroid production in tumour Leydig cells by lutropin results in increased initiation of translation of a relatively stable (see above) mRNA(s) (i.e. the mRNA(s) coding for the rapidly-turning-over protein(s)). Increased initiation of translation might involve actions of lutropin-dependent phosphoproteins of 24000 Da (eIF-4E?) and 33000 Da (ribosomal protein S6). Fig. 4.6 shows in drawing specific initiation of translation under the influence of lutropin.

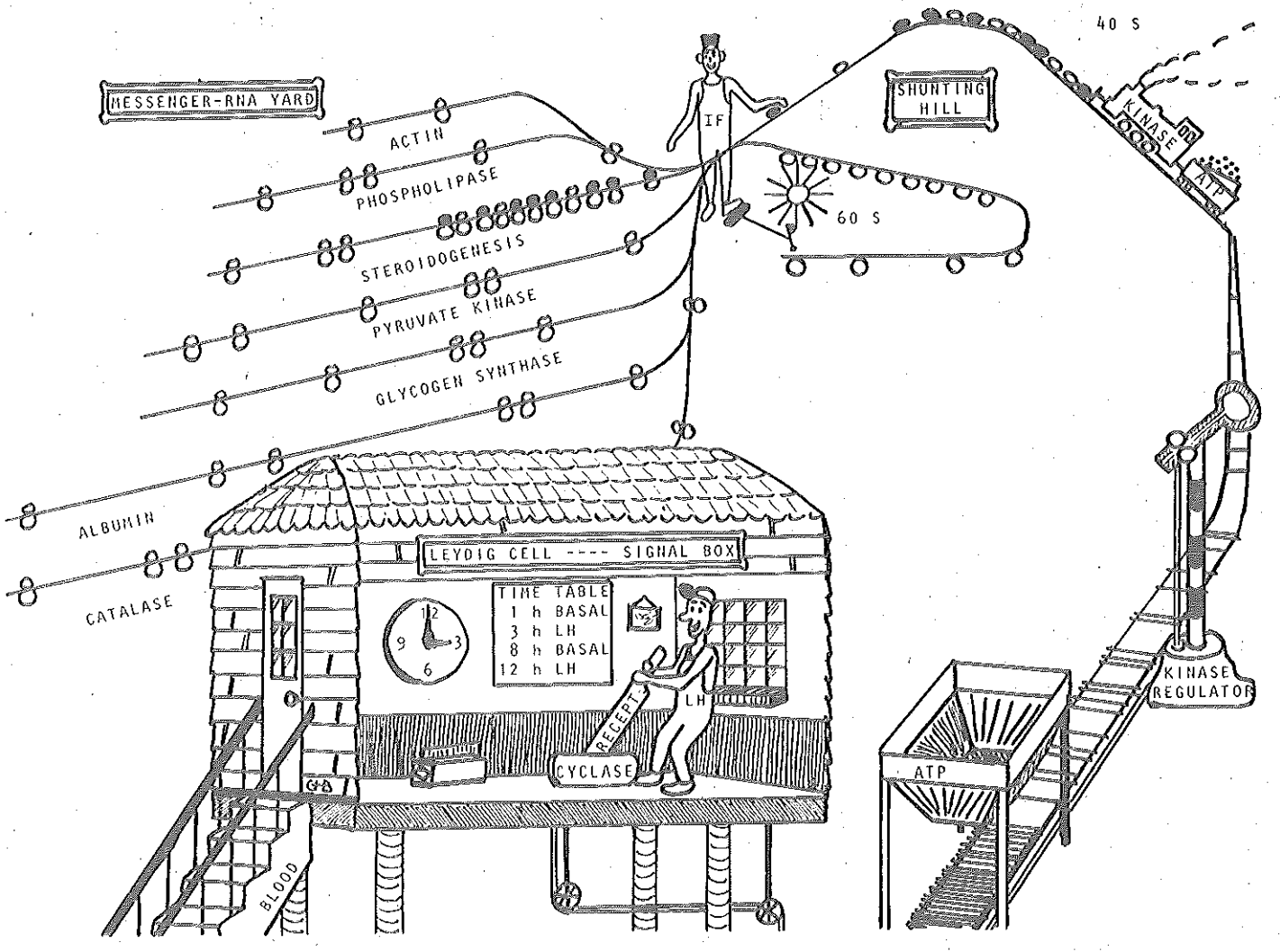
4.6. Conclusions

The similarities in kinetics of increased pregnenolone production and increased (de)phosphorylation of specific proteins under different experimental conditions suggest that lutropin-dependent phosphoproteins could be essential for regulation of steroidogenesis. None of the lutropin-dependent phosphoproteins (see: Table 4.1) was localized inside

Table 4.1. Lutropin-dependent phosphoproteins in tumour Leydig cells.

Molecular mass in daltons	Localization	Significantly phosphorylated
17000	nucleus	within 5 min
20000 *	cytosol	n.d.
22000	cytosol	within 5 min
24000	microsomes	within 5 min
33000	microsomes	within 5 min
43000	cytosol	n.d.
57000	microsomes/cytosol	within 5 min
76000	cytosol	n.d.

n.d., not determined; * protein is dephosphorylated.


MESSENGER-RNA YARD
ACTIN
PHOSPHOLIPASE
STEROIDOGENESIS
PYRUVATE KINASE
GLYCOGEN SYNTHASE
ALBUMIN
CATALASE
IF
40 S
60 S
SHUNTING HILL
KINASE
ATP
LEYDIG CELL ---- SIGNAL BOX
TIME TABLE
1 h BASAL
3 h LH
8 h BASAL
12 h LH
LH
RECEPT.
CYCLASE
GS
BLOOD
ATP
KINASE REGULATOR

mitochondria, and a possible regulation of mitochondrial cholesterol side-chain cleavage activity by these phosphoproteins appeared to be indirect. Phosphoproteins of 20000, 43000 and 76000 Da may be related to the microfilaments; phosphoproteins of 24000 and 33000 Da may be involved in specific protein synthesis.

Fig. 4.6. (facing page) A visualization of the proposed specific effect of lutropin (LH) on protein synthesis. Lutropin sets the green signal for phosphorylation of 40 S ribosomal subunits (the blackened ones), which are pushed over the hill by protein kinase and onto the steroidogenesis mRNA-track by Mr. Initiation Factor (IF) (see also: section 4.5).

Chapter 5

GENERAL DISCUSSION

5.1. Introduction

The basic biochemical mechanism underlying steroid production, cholesterol side-chain cleavage, appears to be similar in all cell types known to produce steroids, i.e. adrenal cells, testis cells, ovarium cells. Steroid production may vary to a certain degree between the steroidogenic cell types in relation to matters such as: the amount of steroids produced per unit time, the contribution of lipoproteins to increase the amount of cellular cholesterol, the involvement of cholesterol ester hydrolase. Irrespective of these differences, ideally, any model delineating the way in which steroid production occurs should be applicable to all steroidogenic cell types.

This general discussion is an attempt to integrate the results which were described in the preceding chapters, and additional literature data in a general model on the acute control of steroid production. Several aspects of hormone-dependent steroid production (besides those involving cyclic AMP and Ca^{2+}) have been described in the literature. It is presently difficult to incorporate these different aspects of hormone-induced changes in steroidogenic cells in a model on acute regulation of steroid production, because of the as yet unknown physiological significance and the long-term character of some of these aspects. Some examples are: a change in the cellular membrane potential in adrenal cells stimulated with corticotropin (Lymangrover et al., 1982); the involvement of cyclic GMP (Nambi et al., 1982); specific protein synthesis after long-term stimulation (> 1 h) with hormone (Janszen et al., 1977; 1978a,b; Nakamura et al., 1978; Dazord et al., 1979, 1981; Durwood et al., 1980; Dubois et al., 1981; Losier & YoungLai, 1981; Rowe et al., 1981; YoungLai & Osoko, 1982); the reported presence of a cyclic AMP-dependent protein kinase inside mitochondria (Dimino et al., 1981); the involvement of prostaglandins (Grotjan et al., 1978; Haour et al., 1979; Matsuoka et al., 1980).

The cholesterol side-chain cleavage enzyme is present in the inner-mitochondrial membrane. Increased steroid production appears to result from an increased availability of cholesterol for the cholesterol side-chain cleavage enzyme (cf. sections 2.2 and 2.3). The presently described hypothesis of steroid production (section 5.4) is concerned with a possible way to increase acutely the availability of cholesterol for the cholesterol

side-chain cleavage enzyme. It will be understood that the hypothesis of steroid production is mainly theoretical and will require experimental verification.

5.2. Possible role for the individual lutropin-dependent phosphoproteins

In previous sections a possible role for lutropin-dependent phosphoproteins was described in relation to microfilaments (cf. sections 2.3 and 4.5) and specific protein synthesis (cf. section 4.5). Lutropin-dependent phosphoproteins of 20000, 43000 and 76000 Da could be related to microfilaments in being similar to: myosin light-chain (the 20000 Da protein); actin (the 43000 Da protein); myosin light-chain kinase (the 76000 Da protein). Lutropin-dependent phosphoproteins of 24000 and 33000 Da might be involved in specific protein synthesis. The 24000 Da phosphoprotein might be similar to initiation factor eIF-4E, whereas the 33000 Da phosphoprotein appears to be ribosomal protein S6.

The lutropin-dependent phosphoprotein of 57000 Da probably is the regulatory subunit of the type II cyclic AMP-dependent protein kinase (Cooke et al., 1979d). Any suggestion concerning the possible role for the lutropin-dependent phosphoproteins of 17000 and 22000 Da would be merely speculation. Interesting aspects of these two phosphoproteins concern the prominent localization in the nuclear fraction (the 17000 Da protein; DNA-bound or membrane-bound?) and in the cytosol fraction (the 22000 Da protein) isolated from tumour Leydig cells, as well as the rather prominent appearance of the respective phosphorylated protein bands observed in the autoradiograms (relatively large amounts present?).

A summary of the above-mentioned possible roles for the lutropin-dependent phosphoproteins is presented in Table 5.1. In agreement with the observations for tumour Leydig cells, Leydig cells isolated from immature and mature rat testes also showed lutropin-dependent phosphorylation of proteins of 17000, 33000, 57000 and 76000 Da (Appendix Paper II) and dephosphorylation of a 20000 Da protein (unpublished observations).

No lutropin-dependent phosphoprotein could be demonstrated in the mitochondrial fraction isolated from tumour Leydig cells, which indicates that regulation of the mitochondrial side-chain cleavage enzyme via specific phosphoproteins probably occurs indirectly. Conflicting results have been described regarding phosphorylation of the cholesterol side-chain cleavage enzyme (cf. Appendix Paper I; Defaye et al., 1982). Acute regulation of steroid production by lutropin via specific phosphoproteins related to microfilaments and protein synthesis appears attractive on the basis of the results described in Chapter 4, in combination with results

Table 5.1. Possible physiological role for lutropin-dependent phosphoproteins.

Molecular mass in daltons	Localization	Possible function
17000	nucleus	DNA-bound or membrane-bound
22000	cytosol	protein phosphatase inhibitor-1 troponin-I calcium-efflux from mitochondrion
24000/33000	microsomes	specific protein synthesis: 24000, cap-binding protein 33000, ribosomal protein S6
57000	microsomes/ cytosol	regulatory subunit of cyclic AMP-dependent protein kinase
43000/76000 20000*	cytosol	present in microfilaments: 43000, actin 76000, myosin light-chain kinase 20000, myosin light-chain

*, protein is dephosphorylated.

described in the literature. The involvement of the nucleus in acute regulation of steroid production in the presence of lutropin appears improbable (cf. section 4.5), although further experiments are necessary to firmly prove this notion.

5.3. Molecular aspects of steroid production

5.3.1. Polyphosphorylated lipids and steroid production

The cholesterol side-chain cleavage enzyme is (partly) embedded in the inner-mitochondrial membrane. It is generally known that the activity of membrane-bound enzymes is dependent on the fluidity of the membrane. Membrane fluidity is determined mainly by the ambient temperature and by the type and degree of saturation of the phospholipids present in the membrane.

Phospholipids appear to be involved in regulation of enzymes present in membranes (reviews by Berridge, 1981; Shukla, 1982). It has been shown that excess phosphatidyl ethanolamine and phosphatidyl choline inhibit cytochrome P-450 activity (Mason & Boyd, 1971; Ungar et al., 1973). Adrenal mitochondrial membranes comprise relatively large amounts of both

Table 5.2. Lipid composition of bovine adrenocortical mitochondria.

	Wang et al., 1974	Hall et al., 1979b
Phosphatidyl choline	approx. 42%	approx. 38%
Phosphatidyl ethanolamine	39%	57%
Cardiolipin	13.5%	6%
Sphingomyelin		2%
Phosphatidyl inositol	4%	

% indicates relative amount by weight.

phosphatidyl ethanolamine and phosphatidyl choline (Table 5.2; cf. Wang et al., 1974) which might be the consequence of the presence of cytochrome P-450-11β (cf. Wang et al., 1974). It is possible, therefore, that the activity of cholesterol side-chain cleavage is repressed by the high amounts of phosphatidyl ethanolamine and phosphatidyl choline. In contrast, a polyphosphorylated lipid (i.e. cardiolipin) appears to act as a positive modulator of cholesterol side-chain cleavage activity. Cardiolipin (1,3-diphosphatidyl glycerol) appears to interact with the cytochrome P-450, thereby stimulating steroid production (Lambeth, 1981). Half-maximal stimulation of cholesterol side-chain cleavage activity was observed when membranes with the reconstituted cholesterol side-chain cleavage enzyme contained 10-20% (by weight) of cardiolipin. Since the adrenal mitochondria contain only small amounts of cardiolipin (see: Table 5.2), the enzyme is probably not fully activated (Lambeth, 1981).

In addition to the above-mentioned, it was recently suggested that polyphospholipids might be involved in corticotropin regulation of steroid production by the cholesterol side-chain cleavage enzyme. Addition of cardiolipin to rat adrenal mitochondria could mimic corticotropin stimulation of pregnenolone production (Farese & Sabir, 1979), and it was shown that corticotropin acutely increased formation of polyphospholipids in rat adrenal (Farese et al., 1979). The specific phospholipids involved were: diphosphoinositide (phosphatidyl inositol-4'-phosphate) and triphosphoinositide (phosphatidyl inositol-4',5'-diphosphate). Addition of diphosphoinositides to mitochondria, or even to cells, resulted in stimulation of steroid production (Farese et al., 1980a).

Studies with rat tumour Leydig cells in our laboratory did not indicate activation of polyphospholipid synthesis after addition of lutropin (Terpstra et al., 1983). Moreover, addition of cardiolipin or

diphosphoinositide to isolated mitochondria resulted in an increased pregnenolone production of approx. 25% and 50% resp., whereas triphosphoinositide had no effect (Terpstra et al., 1983). Similar experiments with mitochondria isolated from rat adrenal showed much higher stimulated pregnenolone production (approx. + 70%) after addition of cardiolipin or triphosphoinositide, whereas addition of diphosphoinositide to mitochondria resulted in an increase in pregnenolone production of approx. 20% (Terpstra et al., 1983). In contrast, Lowitt et al. (1982) reported a 2-3 fold increased amount of polyphosphoinositides in rat Leydig cells treated with lutropin.

Similar to the results obtained by Terpstra et al. (1983), no increased amount of polyphospholipids could be detected after stimulation of rat granulosa cells with follitropin (Naor & Yavin, 1982) or rat corpus luteum with human choriogonadotropin (Tanaka & Strauss III, 1982). With respect to the latter investigation, Farese's group has described that stimulation of bovine luteal cells with lutropin also resulted in insignificant changes in the amounts of polyphosphoinositides (Davis et al., 1981).

Most of the observations on stimulatory effects of hormones on polyphospholipid synthesis and of polyphospholipids on steroid production originate from the group of Farese, and mainly concern rat adrenal. Other investigators using ovarian or testicular cells could detect either no effect or only a marginally increased amount of polyphospholipids. These results could reflect that a hormone-dependent increase of polyphospholipids in steroidogenic cells is limited to adrenal cells (related to the presence of cytochrome P-450-11β?) and may constitute a specialized feature of hormonal regulation of steroid production.

5.3.2. Calcium ions and steroid production

Hormone-dependent steroid production can be increased by the presence of calcium ions (Van der Vusse et al., 1975; Janszen et al., 1976). The involvement of calcium ions in hormone-dependent steroid production has been studied by depletion of calcium ions in the incubation medium, by inhibiting calmodulin (the protein which enables calcium regulation of enzyme activity), or by using calcium ionophores. Depletion of calcium ions has been employed in studies with rat testis Leydig cells (Janszen et al., 1976), rat adrenocortical cells (Trzeciak & Mathé, 1981) and swine granulosa cells (Veldhuis & Klase, 1982a). It was shown that depletion of calcium ions did not affect basal steroid production, whereas hormone-

stimulated steroid production was inhibited (Janszen et al., 1976; Trzeciak & Mathé, 1981; Veldhuis & Klase, 1982a). Moreover, it appears that calcium ions are not required for activation of cyclic AMP-dependent protein kinase (Janszen et al., 1976) or for the conversion of exogenously supplied pregnenolone (Lin et al., 1980; Farese et al., 1981; Veldhuis & Klase, 1982a).

Transport of cholesterol to mitochondria and side-chain cleavage of cholesterol by isolated mitochondria were increased after fusion of Ca^{2+}/calmodulin-containing liposomes with mouse adrenal tumour cells (Hall et al., 1981a) and rat testis Leydig cells (Hall et al., 1981b). In those studies it was suggested that Ca^{2+}/calmodulin may be involved in regulation of transport of cholesterol to mitochondria, a process stimulated by corticotropin and lutropin.

Calmodulin regulation of various enzymes and cellular processes has been reviewed recently (Stoclet, 1981; Cheung, 1982). The effect of Ca^{2+}/calmodulin on microtubules may be of great importance for the supply of cholesterol to mitochondria. Clark & Shay (1981) have observed some kind of granular storage of cholesterol in microtubular structures. Stimulation of murine adrenal cells and rat Leydig cells with corticotropin or dibutyryl cyclic AMP, resulted in dissociation of these microtubular cholesterol-containing granules.

Activation of a Ca^{2+}-dependent protein kinase may also be involved in steroidogenic cells. The lutropin-dependent phosphoprotein of 43000 Da has a molecular mass similar to actin and might be considered as a Ca^{2+}-dependent phosphoprotein on the basis of the results obtained by Nosé & Schulman (1982). They have demonstrated the presence of a Ca^{2+}-dependent protein kinase in bovine brain cytosol which is activated by Ca^{2+} plus calmodulin. The most prominent substrate was a polypeptide with a molecular mass of 45000 Da. The rather prominent phosphorylation of the 45000 Da protein (cf. Nosé & Schulman, 1982) and the lutropin-dependent phosphoprotein of 43000 Da (cf. Fig. 4.4), may indicate the presence of many of these proteins in the cytosol in accordance with the idea that these proteins may be similar to actin.

Several reports have described the inhibitory action of the calcium ionophore A23187 on hormone-dependent steroid production (Lin et al., 1980; Farese et al., 1981; Veldhuis & Klase, 1982b). The reported inhibition of hormone-dependent steroid production by A23187 may be the consequence of increased intracellular calcium concentration, since the concentration of calcium ions in the incubation medium (e.g. 2.5 mM, cf. Appendix Paper I; in blood: 1.25 mM, Williamson et al., 1981) is at least 1000 times higher than the concentration in the cytosol (0.3 µM, Becker et al., 1980). High

intracellular calcium concentrations may account for: 1. inhibition of protein synthesis observed in the presence of A23187 (Farese et al., 1981; Veldhuis & Klase, 1982b), 2. possible damage of mitochondria (cf. Hunter & Haworth, 1979a,b; see also: Simpson, 1979). Moreover, high intracellular calcium concentrations might interfere with cholesterol transport to mitochondria (cf. section 2.3), since calcium ions can make microfilaments more rigid (cf. e.g. review by Stoclet, 1981).

Stimulation of liver cells with hormones acting via cyclic AMP (e.g. glucagon, β-adrenergic agents) or some other factor (e.g. α-adrenergic agents, vasopressin, angiotensin II) (cf. review by Williamson et al., 1981) results in a redistribution of cellular calcium between mitochondria (Chen et al., 1978; Babcock et al., 1979; Blackmore et al., 1979; Murphy et al., 1980) and the endoplasmic reticulum (Waltenbaugh & Friedmann, 1978; Taylor et al., 1980), with possible extrusion of Ca^{2+} out of the cells (cf. Williamson et al., 1981). A hormone-dependent calcium pool in mitochondria is considered in the hypothesis on steroid production (section 5.4) to account for the calcium involvement in hormone-dependent steroid production (see above). It is presently unknown in what way the intracellular redistribution of calcium ions might be explained (cf. Whiting & Barritt, 1982, and refs. therein). In the hypothesis on steroid production (section 5.4) a possible role of microfilaments in Ca^{2+}-release from mitochondria is indicated.

5.3.3. Protein synthesis and steroid production

Basal steroid production in rat Leydig cells (Cooke et al., 1975) and rat adrenal cells (Gill, 1972; Farese et al., 1980a) is not inhibited by inhibitors of protein synthesis, such as cycloheximide and puromycin. However, basal steroid production in tumour Leydig cells is inhibited by inhibitors of protein synthesis, possibly owing to a slightly stimulated steroid production under basal condition (cf. Appendix Paper II).
Hormone-stimulated steroid production (i.e. with lutropin or corticotropin) is inhibited by inhibitors of protein synthesis (Garren et al., 1965; Schulster et al., 1974; Cooke et al., 1975; Appendix Paper II). The degree of inhibition of protein synthesis appears to parallel the degree of inhibition of corticotropin-stimulated corticosterone production (Schulster et al., 1974) or lutropin-stimulated testosterone production (Cooke et al., 1975) (see also: Appendix Paper IV). Apparently, a protein factor(s) is required for hormonal stimulation of steroid production. It has been suggested that this protein(s) may increase the availability of cholesterol for the cytochrome P-450 (see: section 2.2).

The kinetics of inhibition of hormone-dependent steroid production by inhibitors of protein synthesis indicated the following half-lifes for the putative protein factor(s) involved: 8 min (Garren et al., 1965); 2-4 min (Schulster et al., 1974); 13 min (Cooke et al., 1975); 3.5 min (Farese et al., 1980b); 6 min (Appendix Paper II). Garren et al. (1965) introduced the name of "rapidly-turning-over protein(s)" to denote the protein factor(s) required for hormone-dependent steroid production.

Lutropin-dependent phosphoproteins may be involved in regulation of synthesis of the rapidly-turning-over protein(s) (cf. section 4.5), i.e. lutropin-dependent phosphoproteins may stimulate either DNA transcription or mRNA translation, or both processes, to increase the amounts of the rapidly-turning-over protein(s).

Experiments with actinomycin D to inhibit RNA synthesis showed that inhibition of (m)RNA synthesis does not impair corticotropin-dependent (Garren et al., 1965) or lutropin-dependent (Cooke et al., 1979b) steroid production. The mRNA(s) coding for the rapidly-turning-over protein(s) appears to be rather stable (Garren et al., 1965; Cooke et al., 1979b; Losier & YoungLai, 1981; section 4.5). Thus, an acute effect of lutropin-dependent phosphoproteins on transcription appears not required for increased steroid production.

Several studies indicate that overall protein synthesis in steroidogenic tissues or cells is not increased immediately after addition of hormone (Garren et al., 1965; Cooke et al., 1975; YoungLai & Osoko, 1982; Appendix Paper II). Regulation of protein synthesis appears to occur at the initiation of translation (Lodish, 1976; Hunt, 1980a; Snoek et al., 1981; Monier & Le Marchand-Brustel, 1982). Increased rate of initiation is involved in the effects of insulin on overall protein synthesis (Monier & Le Marchand-Brustel, 1982), and in the effect of dibutyryl cyclic AMP on specific protein synthesis (Snoek et al., 1981). Lutropin-dependent phosphoproteins of 24000 and 33000 Da may be involved in specific protein synthesis (cf. section 4.5). Phosphorylation of initiation factor eIF-2 is clearly involved in regulation of haemoglobin synthesis (see reviews by: Austin & Clemens, 1980; Hunt, 1980b). The lutropin-dependent phosphoprotein of 24000 Da might be similar to initiation factor eIF-4E (cf. section 4.5).

The lutropin-dependent phosphoprotein of 33000 Da most probably is ribosomal protein S6. Up to five phosphorylated derivatives of protein S6 (with an increasing number of phosphate groups) could be distinguished using electrophoresis of phosphorylated ribosomal proteins (cf. Leader, 1980). In regard of the proposed role for protein S6 in specific protein synthesis in the presence of lutropin (cf. section 4.5) it is interesting

to note that it has been suggested that specific alterations in phosphorylation of the S6 protein could selectively influence the ability of the ribosomal population to translate some specific mRNAs in the (rat) brain (Roberts & Morelos, 1980). However, up to now, no report has described selectivity of ribosomes for mRNAs on the basis of the presence of a particular protein S6, i.e. a protein S6 with a specific number of phosphate groups. Nevertheless, ribosomes with phosphorylated derivatives of protein S6 appear to be used preferentially during the formation of initiation complexes in HeLa cells (Duncan & McConkey, 1982a,b) and fibroblasts (Thomas et al., 1982), whereas estimation of the protein synthetic activity of ribosomes differing in the extent of phosphorylation of ribosomal protein S6 showed no significant difference in the in-vitro ability to synthesize polyphenylalanine (Leader et al., 1981).

It is still puzzling in what way a specific effect on protein synthesis induced by protein hormones (e.g. glucagon, lutropin), acting via cyclic AMP, can be accomplished. It is known that mRNAs differ in the efficiency of translation due to differences in the relative rates of initiation (Lodish, 1976; Kozak, 1978). This may have something to do with a competition of mRNAs for ribosomes or some other factor(s) involved in initiation (e.g. Walden et al., 1981; Hall et al., 1982). On the basis of the present knowledge on initiation of translation, it appears that a differential utilization of mRNAs might be explained by a different requirement of the various mRNAs for some specific initiation factors (including the 24000 Da cap-binding protein or eIF-4E), as proposed by Voorma (1983) (cf. also: Hall et al., 1982). These specific initiation factors are required for unfolding (i.e. melting of the secondary structure; cf. Voorma, 1983) of the cap or leader-sequence on the 5'-end of mRNAs in order to increase the accessibility of mRNAs for ribosomes. In this respect one may consider complexes of a specific mRNA and proteins to constitute ribonucleoprotein particles.

Iatrou et al. (1978) have described the presence and non-utilization of the protamine mRNA during early stages of spermatogenesis in the rainbow trout. After meiosis resulting in spermatid formation, translation of the protamine mRNA can be detected. To account for the retarded translation of the protamine mRNA, Iatrou et al. suggested storage of the protamine mRNA in ribonucleoprotein particles. The latter notion may hold also for the translational differences in spermatogenic cells of mice which showed synthesis of different proteins in the different spermatogenic cells (Fujimoto & Erickson, 1982). Perhaps utilization of a specific mRNA is related to the inhibitory action of cytoplasmic RNA molecules (e.g. Pluskal & Sarkar, 1981; Rosen et al., 1981).

Summarizing, hormone-dependent steroid production appears to involve synthesis of a protein factor(s) which is a rapidly-turning-over protein(s). Synthesis of this protein factor(s) in the presence of hormone might reflect a specific effect on translation via increased initiation of translation of its stable mRNA(s). It is presently unknown how a specific effect on translation could be accomplished. Specific phosphoproteins might be involved in the release of the stable mRNA(s) from ribonucleoprotein particles or in changing its secondary structure, as well as other factors, to explain the proposed specific effect on protein synthesis.

5.4. A hypothesis on the regulation of basal and hormone-dependent steroid production

In general, basal steroid production is not inhibited by inhibitors of protein synthesis. The amount of cholesterol consumed by adrenal cytochrome P-450 without corticotropin stimulation could be accounted for merely by the amount of cholesterol present in the inner-half of the inner-mitochondrial membrane (i.e. the steroidogenic amount of cholesterol; Kimura, 1981). Basal pregnenolone production might be the consequence of a redistribution of cholesterol in the inner-mitochondrial membrane via flip-flop motion between the outer and inner halves of the inner-mitochondrial membrane (Fig. 5.1).

Stimulation of (tumour) Leydig cells with lutropin elicits several intracellular responses which may result in acute and, possibly, long-term regulation of the steroidogenic machinery. Considering the possible physiological role for the individual lutropin-dependent phosphoproteins (cf. section 5.2), the following sequence of events may be involved in acute regulation of steroid production (Fig. 5.2).

Binding of lutropin to its receptor on the cell membrane results in increased formation of cyclic AMP. Cyclic AMP diffuses from the cell membrane into the cytosol and binds to the regulatory subunit of cyclic AMP-dependent protein kinase which is attached to microtubules. Subsequently, autophosphorylation of the regulatory subunit of the kinase takes place with ensueing release of the catalytic subunit. This catalytic subunit starts off to phosphorylate proteins in its vicinity, providing a very local ("compartmentalized") effect of cyclic AMP. If more lutropin is bound to its receptors, more cyclic AMP will be made and, as a consequence, more protein kinase molecules will be activated. This could result (via phosphorylation of proteins) in extension of the part of the cytosol which may participate in the actual cellular response to lutropin (see also: Hayes & Brunton, 1982).

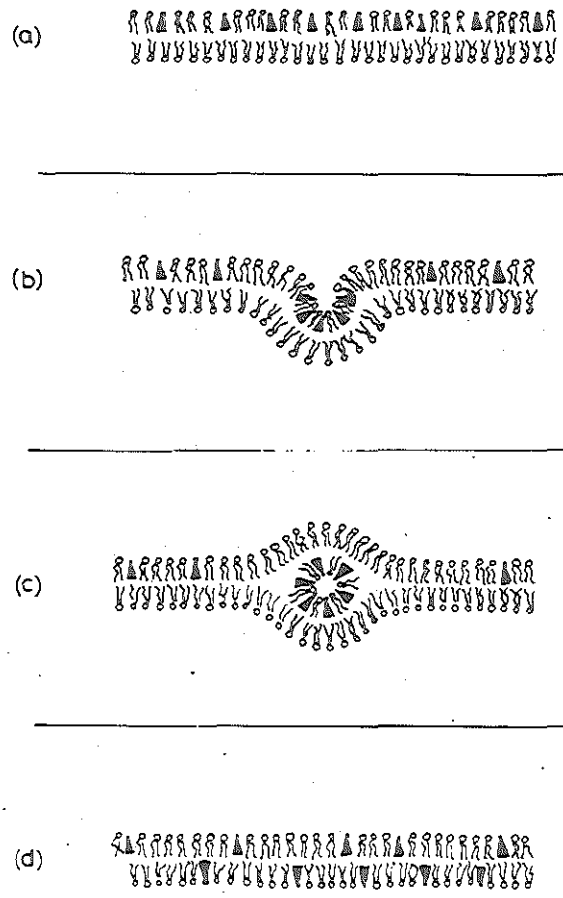


Fig. 5.1. Flip-flop mechanism of "non-bilayer" lipid (▲) as proposed by Cullis & De Kruijff (1978).

The activated kinase may cause changes in phosphorylation of the microfilament proteins of 76000 and 20000 Da. Dephosphorylated 20000 Da myosin light-chains induce relaxation of the microfilament proteins (e.g. Adelstein et al., 1982; Somlyo et al., 1982). The resulting changes in the cytoskeleton may influence the membrane characteristics of mitochondria, since they are attached to the cytoskeleton. A possible lutropin-dependent Ca^{2+}-efflux from mitochondria (cf. e.g. Babcock et al., 1979) may counteract the cyclic AMP-induced relaxation phenomenon, since Ca^{2+}/calmodulin-dependent activation of myosin light-chain kinase will cause rephosphorylation of the 20000 Da phosphoprotein (cf. Cheung, 1982). Perhaps, one should envisage local cycles of relaxation-contraction of the microfilament proteins depending on the local concentrations of cyclic AMP

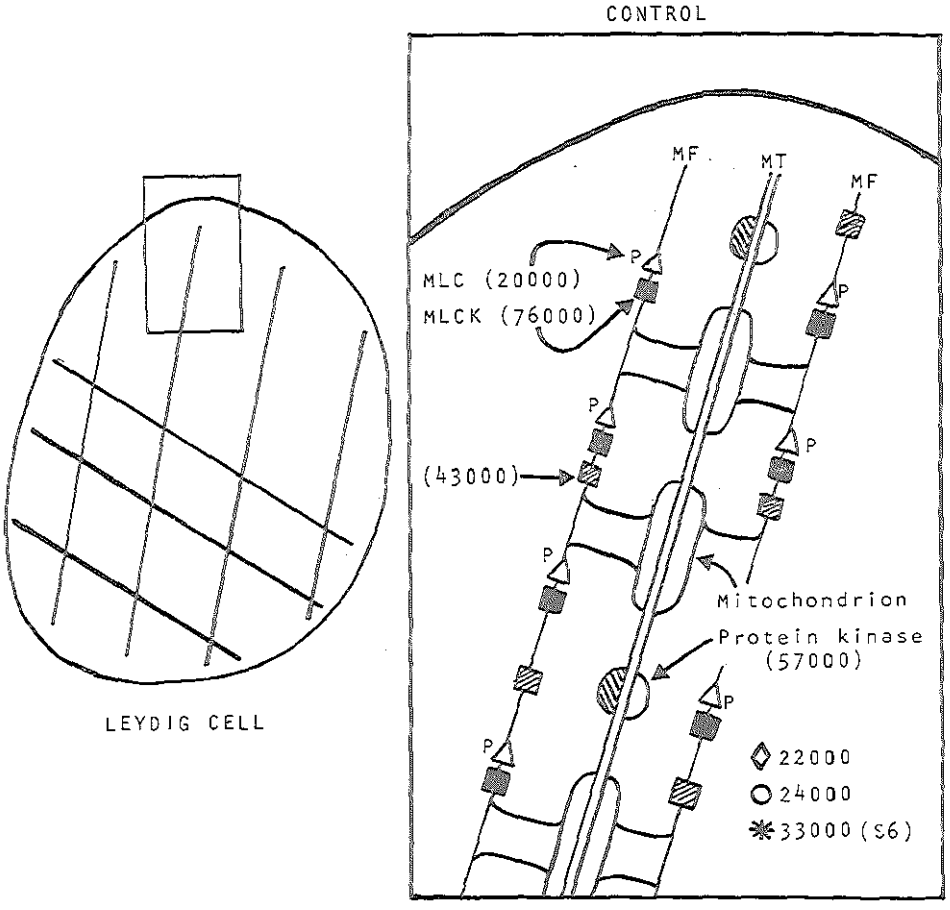
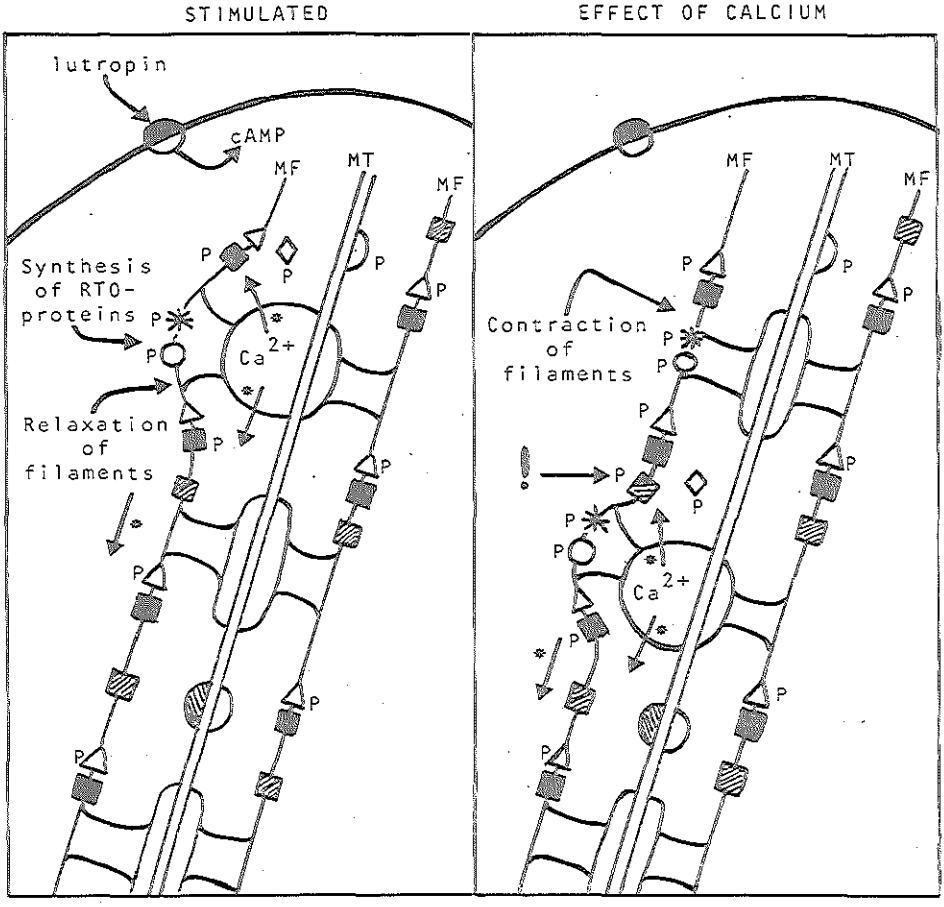


Fig. 5.2. (this and facing page) A hypothetical model on regulation of steroid production. Abbreviations used: MF, microfilament; MT, microtubule; protein kinase, cyclic AMP-dependent protein kinase (hatched part, catalytic subunit). Symbols and corresponding numerical values denote lutropin-dependent phosphoproteins. P, denotes phosphorylated protein. The Figure does not indicate the exact spatial arrangement and changes of the various components. The arrows marked (*) in the two frames on the right indicate the direction of the relaxation process induced by cyclic AMP, as well as the Ca^{2+}-efflux from the mitochondrion. The altered mitochondrial conformations drawn in the two frames on the right denote mechanically distorted mitochondria.
Binding of lutropin to the Leydig cell causes formation of cyclic AMP which activates protein kinase. Phosphorylation of



specific proteins results in relaxation of microfilaments and in specific protein synthesis. Calcium ions leaking from the mitochondria as a result of the microfilament relaxation, induce contraction of the microfilaments. Repetitive cycles of relaxation-contraction may induce microfilament movements enabling transport of cholesterol to the mitochondria. Cholesterol is transferred to the inner-half of the inner-mitochondrial membrane with the aid of rapidly-turning-over (RTO) protein. Effects elicited by Ca^{2+}-ions (including Ca^{2+}/calmodulin-dependent phosphorylation of proteins; see: frame, far right, note of exclamation) and phosphatases abolish the cyclic AMP-induced effects. The role of the 22000 Da phosphoprotein is indistinct (for discussion, see: section 5.4).

and Ca^{2+} (see also: Adelstein et al., 1982). A relaxation-contraction process might transiently increase the permeability of the mitochondrial membranes for calcium ions, thereby leaking out of the mitochondria. Moreover, increased membrane permeability might also favour mitochondrial uptake of cholesterol which is transported to the mitochondria by microfilaments (e.g. Crivello & Jefcoate, 1979). These acute effects will cause an increase in the amount of cholesterol present in mitochondria, and cholesterol could by-pass the time-consuming flip-flop phenomenon (cf. Kimura, 1981) in e.g. the inner-mitochondrial membrane in the presence of rapidly-turning-over proteins (cf. e.g. Farese & Prudente, 1978b; section 2.2). Once cholesterol is present on the matrix side of the inner-mitochondrial membrane, its side-chain is being cleaved and pregnenolone is formed (Simpson, 1979).

A specific effect on protein synthesis which may involve the action of the 24000 and 33000 Da lutropin-dependent phosphoproteins (cf. section 5.3.3) results in formation of the rapidly-turning-over protein(s). In this respect, microfilaments may also be involved, as protein synthesis appears to occur during attachment of polysomes to the cytoskeleton (Heuser & Kirschner, 1980; Cervera et al., 1981; Van Venrooij et al., 1981). The presently available evidence suggests that the secondary structure of a mRNA may limit its utilization in translation (Lodish, 1976; Kozak, 1978; Voorma, 1983). Lutropin-dependent phosphoproteins might change the conformation of the stable mRNA(s) coding for the rapidly-turning-over protein(s) to enable its translation.

As a consequence of the microfilament movements (flutters?), cytosol calcium may increase by a pulsatile leakage of calcium ions from mitochondria, and this in turn could adversely affect the postulated relaxation-contraction process, as well as protein synthesis (cf. Discussion in section 5.3.2; increased cytosol calcium involved in desensitization?; cf. e.g. Morgan et al., 1982).

The next important step is the return to basal activity of steroid production when stimulation of cyclic AMP formation ceases. A possible sequence of events enabling restoration of basal activity may include the following steps.

Phosphatases in the cytosol will remove phosphate groups from (lutropin-dependent) phosphoproteins. Removal of the phosphate from the 57000 Da regulatory subunit of cyclic AMP-dependent protein kinase may increase its affinity for binding of the catalytic subunit of protein kinase. When the regulatory subunit and the catalytic subunit are recombined, cyclic AMP-

dependent phosphorylation of proteins will cease. As a net result, phosphorylation of the lutropin-dependent phosphoproteins, and the effects on microfilaments and specific protein synthesis are abolished. The proposed pulsatile Ca^{2+}-efflux from mitochondria will stop, as well as synthesis of the rapidly-turning-over protein(s). Calcium sequestration by mitochondria, enabled by energy generated in the process of oxidative phosphorylation (cf. Williamson et al., 1981), resumes, whereas calcium possibly bound to the endoplasmic reticulum (e.g. Becker et al., 1980; Williamson et al., 1981) will be released to the cytosol to maintain the steady-state cytosol calcium concentration.

Calcium ions present in the cytosol may, in fact, accelerate the return to basal activities by calmodulin-dependent activation of phosphodiesterase (Purvis & Hansson, 1980) and protein kinase (for a review, see: Cheung, 1982). The latter will cause reduction of the amount of cyclic AMP and formation of specific Ca^{2+}-dependent phosphoproteins, besides myosin light-chain (the lutropin-dependent phosphoprotein of 43000?; cf. section 5.3.2).

As the result of the activities of phosphatases, Ca^{2+} and calmodulin, as described above, the effects on specific protein synthesis and microfilaments elicited by lutropin via cyclic AMP will fade away when cyclic AMP formation is stopped.

5.5. Concluding remarks

The presently described hypothesis is an attempt to incorporate the many different observations made with different steroidogenic cell types. Interestingly, reconstitution experiments, showing increased steroid production by isolated mitochondria supplemented with cytosol fractions isolated from lutropin-stimulated testes (Bakker et al., 1978) or corticotropin-stimulated adrenals (Neher et al., 1982), may be difficult to explain on the basis of the hypothesis. Any involvement of microfilaments in this kind of steroid production is difficult to imagine. However, one may still consider stimulatory actions of e.g. cholesterol, rapidly-turning-over protein(s), present in cytosol fractions isolated from hormone-stimulated cells, added to mitochondria with possibly altered membrane characteristics.

The hypothesis described above (section 5.4) includes an important action of Ca^{2+}-ions and microfilaments, besides a specific effect on protein synthesis, in hormonal regulation of steroid production. Cyclic AMP and Ca^{2+}-ions may interact in some kind of concerted mechanism (cf. Adelstein et al., 1982; Giguere et al., 1982) to bring about changes in

microfilaments enabling transport of cholesterol (Crivello & Jefcoate, 1979), synthesis and transport of the rapidly-turning-over protein(s) (cf. section 5.4). The microfilament movements proposed in the hypothesis are supposed to cause a transiently increased permeability of the mitochondrial membranes resulting in a pulsatile leakage ("release") of calcium ions from the mitochondrion which might be sequestered subsequently by the endoplasmic reticulum. Leakage of calcium-ions from mitochondria clearly should involve increased permeability of the inner-mitochondrial membrane as calcium (phosphate) is accumulated in the mitochondrial matrix (cf. Williamson et al, 1981). Ca^{2+}-efflux from mitochondria, compared with Ca^{2+}-uptake, is a relatively slow process, and Ca^{2+}-efflux can be stimulated in some tissues by Na^{+}-ions (Carafoli, 1979). Perhaps the postulated action of phosphorylated proteins in regulation of Ca^{2+}-efflux from mitochondria (section 5.4) may provide a mechanism which accelerates efflux of calcium. It remains to be demonstrated whether this notion is correct and whether Na^{+}-ions may also take part in regulation of the proposed mitochondrial Ca^{2+}-efflux in hormone-stimulated steroidogenic cells.

In the hypothesis, lutropin-dependent phosphoproteins of 17000 and 22000 Da have not been mentioned. Regulation of nuclear activities possibly involving the lutropin-dependent phosphorylation of the 17000 Da protein is as yet difficult to indicate. The lutropin-dependent phosphoprotein of 22000 Da might be involved in regulation of Ca^{2+}-efflux from mitochondria (cf. Fleschner et al., 1982).

To verify the hypothesis, it is essential to determine very accurately the proposed intracellular calcium displacements in steroidogenic cells. A hormone-responsive calcium pool present in mitochondria may be detected by studying the release of $^{45}Ca^{2+}$ from mitochondria preloaded with $^{45}Ca^{2+}$ within the cell, and isolated from control and hormone-stimulated cells. It would appear attractive to study also the proposed involvement of microfilaments in calcium-leakage from mitochondria with inhibitors of microfilament formation.

Future experiments should be designed to detect the intracellular calcium displacements proposed to occur after hormonal stimulation of steroidogenic cells, as well as to isolate and characterize the rapidly-turning-over protein(s) required for hormone-dependent steroid production, to further elucidate the intricate mechanism of steroid production.

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SUMMARY

Steroids made by foetal testis Leydig cells are causing the development of the foetus into an individual with male primary and secondary characteristics. These steroids can be considered as "male-individual-generating-hormones", i.e. androgens. After puberty, the individual reaching his sexually mature state, androgens are also essential for growth of spermatozoa, the male germinal cells, required for sexual reproduction. Synthesis of androgens by testis Leydig cells can be stimulated by a protein hormone, lutropin, originating from the pituitary. In principle, the work described in this thesis is concerned with the elucidation of the mechanism whereby lutropin-stimulation of Leydig cells results in increased synthesis of androgens.

Synthesis of androgens, steroid hormones in general, depends on the activity of several enzymes resulting in production of a great variety of steroids. The rate-limiting step in the whole process of steroidogenesis, the conversion of cholesterol into pregnenolone, is performed by the cholesterol side-chain cleavage enzyme consisting of three different proteins (for details, see Chapter 2). The activity of this enzyme is regulated by lutropin. Activation by protein hormones of specific cellular activities in a great number of different cell types has been shown to involve phosphorylation of proteins. In combination with the amounts of pregnenolone produced, the effects of addition of lutropin to tumour Leydig cells on phosphorylation of proteins has been investigated.

It was shown that addition of lutropin to tumour Leydig cells resulted in a rapid increase in pregnenolone production concomitant with a rapid increase in phosphorylation of seven proteins of 17000, 22000, 24000, 33000, 43000, 57000 and 76000 Da, and in dephosphorylation of a 20000 Da protein (section 4.2). The similar kinetics of increased (de)phosphorylation of proteins and increased pregnenolone production after addition of lutropin, suggested that lutropin-dependent phosphoproteins could be involved in regulation of steroid production. In order to find out if these specific phosphoproteins may directly influence the activity of the cholesterol side-chain cleavage enzyme present in mitochondria, a subcellular fractionation has been performed. However, none of the lutropin-dependent phosphoproteins was present in mitochondrial fractions isolated from tumour Leydig cells (section 4.4). Apparently, regulation of cholesterol side-chain cleavage in mitochondria by specific phosphoproteins occurs indirectly.

The rapid kinetics of (de)phosphorylation of proteins (cf. section 4.2) made it impossible to assess the importance of each individual

phosphoprotein for increased pregnenolone production, as all lutropin-dependent phosphoproteins could be equally involved in regulation of pregnenolone production. Short-term culture of tumour Leydig cells enabled a further study with respect to which lutropin-dependent phosphoproteins correlate best with increased pregnenolone production. During culture of tumour Leydig cells a gradual decrease in response to lutropin stimulation was observed for (de)phosphorylation of proteins and pregnenolone production in spite of an unimpaired cell viability (section 4.3). A significant decrease in activity of cyclic AMP-dependent protein kinase was demonstrated in combination with a significant decrease in lutropin-dependent phosphorylation of the 57000 Da protein (the regulatory subunit of the type II cyclic AMP-dependent protein kinase). During culture of tumour Leydig cells the decreased activity of cyclic AMP-dependent protein kinase may account for the decrease in lutropin-dependent (de)phosphorylation of proteins, and may contribute to the decrease in lutropin-dependent pregnenolone production (section 4.3). Moreover, the combined results suggest that the nuclear 17000 Da lutropin-dependent phosphoprotein may be involved in long-term regulation, and all other lutropin-dependent phosphoproteins in acute regulation of steroid production.

The possible physiological role for the lutropin-dependent phosphoproteins of 20000, 22000, 24000, 33000, 43000 and 76000 Da was further investigated by studying the subcellular localization and the effects of inhibitors of protein synthesis and microfilament formation (sections 4.4 and 4.5). The results obtained may indicate that:

- microsomal phosphoproteins of 24000 and 33000 Da could be involved in synthesis of rapidly-turning-over protein(s) required for lutropin-dependent steroid production;
- cytosol phosphoproteins of 20000, 43000 and 76000 Da could be part of microfilaments which have been reported to enable cholesterol transport through the cell to mitochondria.

The results obtained with tumour Leydig cells have been combined with the results described in the literature on hormone-dependent regulation of steroid production to constitute a hypothesis on regulation of steroid production (section 5.4). In short, it is proposed that binding of hormone (corticotropin, follitropin or lutropin) to a steroidogenic cell results in activation of cyclic AMP-dependent protein kinase causing increased phosphorylation of specific proteins. These specific phosphoproteins, in turn, give rise to microfilament movements (which involve Ca^{2+}-efflux from mitochondria), synthesis of rapidly-turning-over protein(s), as well as some effect on the cell nucleus. The combined actions of microfilaments and

rapidly-turning-over protein(s) increase the availability of cholesterol for the mitochondrial cholesterol side-chain cleavage enzyme, resulting in increased steroid production, whereas effects on nuclear processes might be involved in long-term regulation of the steroidogenic activity of the cell (Figs. 1.2 and 5.2).

SAMENVATTING

De ontwikkeling van een foetus tot een individu met mannelijke primaire en secundaire geslachtskenmerken wordt veroorzaakt door steroïdhormonen, gemaakt door Leydig cellen die gevormd zijn in de foetale testis. Deze steroïdhormonen zou men kunnen beschouwen als "mannelijk-individu-genererende hormonen", te weten androgenen. Na het bereiken van het sexueel volwassen stadium, d.w.z. na de puberteit, zijn androgenen eveneens essentieel voor de groei van de spermatozoa, de mannelijke zaadcellen, die nodig zijn voor geslachtelijke voortplanting. De synthese van androgenen in testis Leydig cellen kan gestimuleerd worden door een eiwithormoon, het lutropine, dat gemaakt wordt in de hypofyse. Het in dit proefschrift beschreven werk beoogt een bijdrage te leveren tot de opheldering van het mechanisme waardoor stimulatie van Leydig cellen met lutropine resulteert in toegenomen synthese van androgenen.

De synthese van androgenen, en van steroïdhormonen in het algemeen, berust op de aktiviteit van verscheidene enzymen, waardoor veel verschillende steroïdhormonen kunnen worden gevormd. De snelheidsbepalende stap in het proces van de steroïdogenese, de vorming van pregnenolon uit cholesterol, wordt gekatalyseerd door het cholesterol zijketen-splitsend enzym dat uit drie verschillende eiwitten bestaat (voor details, zie Hoofdstuk 2). De aktiviteit van dit enzym wordt gereguleerd door lutropine. In het algemeen blijken eiwithormonen door fosforylering van eiwitten bepaalde cellulaire processen te kunnen beïnvloeden, zoals is aangetoond in een groot aantal verschillende celtypen. Derhalve is onderzocht of stimulatie van de pregnenolonproduktie in (tumor) Leydig cellen door lutropine mogelijk het gevolg is van fosforylering van bepaalde eiwitten.

Stimulatie van tumor Leydig cellen met lutropine resulteerde in een snelle toename in de pregnenolonproduktie en in een snelle toename in fosforylering van zeven eiwitten van 17000, 22000, 24000, 33000, 43000, 57000 en 76000 Da, alsmede in defosforylering van een eiwit van 20000 Da (paragraaf 4.2). De overeenkomstige snelheid van toegenomen (de)fosforylering van eiwitten en van toegenomen pregnenolonproduktie na toevoeging van lutropine gaf aan, dat lutropine-gereguleerde fosfo-eiwitten betrokken konden zijn bij de regulatie van de steroïdproduktie. Om na te gaan of deze bijzondere fosfo-eiwitten een mogelijk direct effekt hebben op de aktiviteit van het cholesterol zijketen-splitsend enzym dat in mitochondriën gelokaliseerd is, is een subcellulaire fraktionering uitgevoerd. Er was echter geen lutropine-gereguleerd fosfo-eiwit aanwezig in mitochondriënfrakties die uit tumor Leydig cellen werden geïsoleerd (paragraaf 4.4). Regulatie van het mitochondriale cholesterol zijketen-

splitsend enzym door bijzondere fosfo-eiwitten geschiedt blijkbaar indirekt.

De snelle (de)fosforylering van de lutropine-gereguleerde fosfo-eiwitten (zie: paragraaf 4.2) maakte het onmogelijk om het belang van elk afzonderlijk fosfo-eiwit voor de toename in pregnenolonproduktie te bepalen, aangezien alle lutropine-gereguleerde fosfo-eiwitten van evengroot belang konden zijn voor de regulatie van de pregnenolonproduktie. Een kortdurende kweek van tumor Leydig cellen maakte verder onderzoek naar het belang van de afzonderlijke lutropine-gereguleerde fosfo-eiwitten voor een toename in de pregnenolonproduktie mogelijk. Tumor Leydig cellen in kweek vertoonden een geleidelijk afnemende respons op lutropine van (de)fosforylering van eiwitten en pregnenolonproduktie, ondanks een onverminderd funktioneren van de cellen (paragraaf 4.3). Er werd een belangrijke daling in aktiviteit van het cyclisch AMP-afhankelijk proteïne-kinase aangetoond, die gelijktijdig optrad met een belangrijke vermindering in lutropine-gereguleerde fosforylering van het 57000 Da eiwit (de regulerende subeenheid van het type II cyclisch AMP-afhankelijk proteïne-kinase). De tijdens de kweek van tumor Leydig cellen afgenomen aktiviteit van het cyclisch AMP-afhankeijk proteïne-kinase verklaart mogelijk de verminderde (de)fosforylering van de lutropine-gereguleerde fosfo-eiwitten, en kan medeverantwoordelijk zijn voor de verminderde respons op lutropine voor wat betreft de pregnenolonproduktie (paragraaf 4.3). Daarenboven wijst het totaal der resultaten op de mogelijke betrokkenheid van het 17000 Da lutropine-gereguleerde fosfo-eiwit (aanwezig in de celkern) bij de lange termijn-regulatie van de steroïdproduktie, en van alle overige lutropine-gereguleerde fosfo-eiwitten bij de acute regulatie van de steroïdproduktie.

De mogelijke fysiologische betekenis van de lutropine-gereguleerde fosfo-eiwitten van 20000, 22000, 24000, 33000, 43000 en 76000 Da werd onderzocht door meer gedetailleerd onderzoek naar de subcellulaire lokalisatie en de effekten van remmers van de eiwitsynthese en van de vorming van microfilamenten (paragrafen 4.4 en 4.5). De resultaten geven aan dat:

- microsomale fosfo-eiwitten van 24000 en 33000 Da betrokken kunnen zijn bij de synthese van eiwitten met een korte levensduur (z.g. "rapidly-turning-over proteins"), die nodig zijn voor de regulatie van de steroïdproduktie door lutropine;
- cytosol fosfo-eiwitten van 20000, 43000 en 76000 Da deel kunnen uitmaken van microfilamenten die betrokken blijken te zijn bij transport van cholesterol naar mitochondria.

Op grond van de resultaten verkregen met tumor Leydig cellen en de

resultaten die beschreven zijn in de literatuur over hormoon-afhankelijke regulatie van steroïdproduktie, is een hypothese voor regulatie van de steroïdproduktie geformuleerd (paragraaf 5.4). In het kort wordt er gesteld, dat binding van een hormoon (corticotropine, follitropine of lutropine) aan een steroïdogene cel resulteert in aktivering van het cyclisch AMP-afhankelijk proteïne-kinase, met als gevolg een toename in fosforylering van bepaalde eiwitten. Deze fosfo-eiwitten veroorzaken op hun beurt bewegingen in microfilamenten (die Ca^{2+}-efflux uit mitochondriën met zich meebrengen), synthese van eiwitten met een korte levensduur, als ook een effekt op de celkern. De gekombineerde werkingen van microfilamenten en de bijzondere, nieuw-gevormde eiwitten, verhogen de beschikbaarheid van cholesterol voor het mitochondriale cholesterol zijketen-splitsend enzym, resulterend in verhoogde steroïdproduktie, terwijl effekten op processen in de celkern betrokken kunnen zijn bij lange termijn-regulatie van de steroïdogene aktiviteit van de cel (Figuren 1.2 en 5.2).

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 9 januari 1954 geboren in Amsterdam. Na het behalen van het H.B.S.-B diploma aan de Christelijke Scholengemeenschap Amsterdam-Oost in 1971 werd begonnen met de scheikunde-studie aan de Vrije Universiteit te Amsterdam. In 1974 werd het kandidaatsexamen S2 behaald en in 1978 het doctoraal examen met als hoofdvak biochemie (prof.dr. R.J. Planta) en als bijvakken organische chemie (dr. J.L. van der Baan) en milieuchemie (dr. P. Bos). Vanaf juli 1978 was hij werkzaam als wetenschappelijk medewerker op de afdeling Biochemie II van de Faculteit der Geneeskunde van de Erasmus Universiteit te Rotterdam, alwaar het in dit proefschrift beschreven werk is verricht.

Appendix Paper I

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Lutropin-dependent protein phosphorylation and steroidogenesis in rat tumour Leydig cells

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Tumour Leydig cells have been incubated in the presence or absence of lutropin (luteinizing hormone, 'LH'). Stimulation of cells with lutropin (1000 ng/ml) in the presence of 1-methyl-3-isobutylxanthine (0.25 mM) resulted in increased steroid production and increased protein phosphorylation. When pregnenolone metabolism was inhibited, basal pregnenolone production was 26.9 ± 7.4 ng/60 min per 10^6 cells; stimulated production was 156.1 ± 39.5 ng/60 min per 10^6 cells (means ± S.D., $n = 4$). Lutropin-dependent phosphorylated proteins of molecular mass 17000, 22000, 24000, 33000 and 57000 Da were detected. A significant increase of [^{32}P]P_i incorporation into these phosphorylated proteins was observed concomitant with the increased pregnenolone production. The occurrence of the phosphoproteins in nuclei, mitochondria and postmitochondrial-supernatant was investigated. The 17000 Da phosphoprotein was found in the nuclear fraction, whereas the 22000, 24000, 33000 and 57000 Da phosphoproteins were localized in the postmitochondrial-supernatant fraction. Of the cholesterol-side-chain-cleavage activity, $80.3 \pm 6.1\%$ (mean ± S.D., $n = 5$) was present in the mitochondrial fraction isolated from tumour Leydig cells, and this activity was 2.5-fold increased when cells had been preincubated with lutropin/1-methyl-3-isobutylxanthine (basal production: 194.6 ± 28.6 ng/30 min per mg of protein; lutropin-stimulated production: 498.8 ± 91.5 ng/30 min per mg of protein; means ± S.D., $n = 3$). The similarities in the kinetics of the phosphorylation of proteins and the pregnenolone production after addition of lutropin/1-methyl-3-isobutylxanthine indicate that the phosphoproteins could be involved in the lutropin-dependent increase in steroidogenesis in tumour Leydig cells. It remains to be demonstrated, however, to what extent the phosphoproteins outside the mitochondria can influence the cholesterol-side-chain-cleavage activity inside the mitochondria.

The effects of lutropin (luteinizing hormone, 'LH') on testis Leydig cells involve an increase in the cyclic AMP concentration (Moyle & Ramachandran, 1973; Rommerts *et al.*, 1973; Podesta *et al.*, 1978), which is followed by activation of cyclic AMP-dependent protein kinase (Rubin & Rosen, 1975; Cooke *et al.*, 1976) and phosphorylation of proteins (Cooke *et al.*, 1977). It is unknown, however, to what extent phosphorylated proteins are involved in the lutropin-dependent increase in steroidogenesis. It has been shown that: (1) the rate-limiting step of steroidogenesis is the conversion of cholesterol into pregnenolone by the cholesterol-side-chain-cleavage-enzyme system inside mitochondria (Simpson, 1979); (2) Leydig-cell mitochondria, isolated after pretreatment of animals with human choriogonadotropin, show increased steroid production (Van der Vusse *et al.*, 1975); (3) cytosol fractions from lutropin-treated rat testes can stimulate the pregnenolone production by mitochondria from non-stimulated rat testes (Bakker *et al.*, 1978); (4) lutropin (100 ng/ml) causes phosphorylation of proteins in adult rat testis Leydig cells of 17000, 57000 and 76000 Da (Cooke *et al.*, 1977). Hence, it is possible that phosphoproteins are essential and could link the action of lutropin at the plasma membrane to the increased steroid production.

We have studied, in tumour Leydig cells, the

Abbreviation used: SDS, sodium dodecyl sulphate.

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correlation between the kinetics of phosphorylation of proteins and steroid production in the presence of lutropin/1-methyl-3-isobutylxanthine, as well as the subcellular localization of the phosphorylated proteins with particular reference to the mitochondria.

Materials and methods

Sheep lutropin (NIH-LH-S18; 1.03 i.u./mg) was a gift from the Endocrinology Study Section of the National Institute of Health, Bethesda, MD, U.S.A. 1-Methyl-3-isobutylxanthine was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. [^{32}P]Orthophosphate (carrier-free) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Cyanoketone (2α-cyano-17β-hydroxy-4,4',17α-trimethylandrost-5-en-3-one), an inhibitor of 3β-hydroxysteroid dehydrogenase activity, and SU-10603 [7-chloro-3,4-dihydro-2-(3-pyridyl)-naphthalen-1(2*H*)-one], an inhibitor of 17α-hydroxylase activity, were kindly donated by Dr. R. Neher from the Friedrich Miescher Institute, Basle, Switzerland. Crude collagenase was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A.

The properties of the tumour Leydig cells were previously described (Cooke *et al.*, 1979*a*). Implantation of tumour tissue was performed by injecting small pieces of tumour subcutaneously into intact male Wistar rats, substrain R_1-Amsterdam, 14–17 weeks of age. Rats were killed 4–7 weeks after injecting the tumour tissue by decapitation after light diethyl ether anaesthesia, and cells were isolated as described previously (Cooke *et al.*, 1979*a*). Cells were incubated under an O_2/CO_2 (19:1) atmosphere in a shaking water-bath (80 cycles/min) at 32°C. Lutropin and 1-methyl-3-isobutylxanthine were added in a concentrated solution and final concentrations were 1000 ng/ml and 0.25 mM respectively.

Kinetic studies of phosphorylation of proteins and pregnenolone production were performed by addition of lutropin/1-methyl-3-isobutylxanthine at different time intervals before the end of the incubation period. For studies of phosphorylation, cells were preincubated for 1 h in Krebs–Ringer buffer (118.3 mM-NaCl/4.75 mM-KCl/25 mM-$NaHCO_3$/1.2 mM-$MgSO_4$/2.5 mM-$CaCl_2$), pH 7.3, without phosphate, containing 0.2% glucose and 0.1% albumin. Incubations were performed with 200 μl suspensions containing 2×10^6 cells without or with lutropin/1-methyl-3-isobutylxanthine. Labelling was started by addition of 50 μCi of [^{32}P]orthophosphate. Incorporation of label was stopped after 1 h by the addition of a cold (4°C) iso-osmotic medium containing 0.125 M-NaH_2PO_4, 82 mM-Tris and 20 mM-NaF, pH 7.3. Cells were centrifuged, resuspended in SDS-containing lysing buffer (0.1 M-glycine/0.1 M-NaCl/10 mM-EDTA/0.1% SDS/10 mM-β-mercaptoethanol/20 mM-NaF) and boiled for 3–5 min. After boiling, the proteins were precipitated by addition of acetone (4:1, v/v), washed with 70% (v/v) ethanol and diethyl ether, and finally dissolved in the SDS-containing sample medium [50 mM-Tris/HCl/10% glycerol (pH 6.8), containing 2% SDS and 1% β-mercaptoethanol]. The amount of protein-bound [^{32}P]orthophosphate was determined after trichloroacetic acid precipitation of a portion of the protein samples on Whatman filters (3 MM Chroma). Precipitates on filters were washed with 70% ethanol and ether, followed by counting of ^{32}P radioactivity on dry filters using methoxytoluene as scintillation fluid. Electrophoresis was performed with SDS/8–15%-polyacrylamide gradient slab gels essentially as described by Laemmli (1970), with a Bio-Rad model 220 dual vertical slab-gel-electrophoresis cell (100 mm × 140 mm × 1.5 mm gels). After electrophoresis the gels were fixed in methanol/water/acetic acid (5:4:1, by vol.) for at least 1 h. Staining of gels was complete in 30–45 min with PAGE Blue 83 (1 g/litre; BDH, Poole, Dorset, U.K.) in ethanol/water/acetic acid (5:11:4m by vol.), containing 1 g of $CuSO_4$/litre. Destaining was achieved by a single wash with methanol/water/acetic acid (30:63:7, by vol.). The gels were dried on a Bio-Rad model-224 gel-slab dryer under continuous heating at 70–80°C.

After being dried, the gels were exposed to Kodak X-ray film SB-5. The autoradiograms obtained were scanned with a Gilford model-2400 spectrophotometer. The correlation between the amount of radioactivity in the gel and the densitogram of the exposed X-ray film was evaluated by using autoradiograms of known increasing amounts of radioactivity. The molecular weights of the proteins were calculated by comparison with the mobilities of the standard proteins present in the Bio-Rad low-molecular-weight-standard mixture.

For studies of pregnenolone production, cells were preincubated for 1 h in Krebs–Ringer buffer, pH 7.3, with 0.2% glucose and 0.1% albumin. Incubations were for 1 h in 500 μl suspensions containing 1×10^6 cells without or with lutropin and 1-methyl-3-isobutylxanthine, in the presence of cyanoketone and SU-10603 at final concentrations of 5 μM and 19 μM respectively. Inhibitors of pregnenolone metabolism and lutropin/1-methyl-3-isobutylxanthine were added simultaneously. Incubations were stopped by the addition 2×2 ml of ethyl acetate, and production of pregnenolone was determined by using a radioimmunoassay for pregnenolone (Van der Vusse *et al.*, 1975).

For studies on the subcellular localization of phosphorylated proteins and cholesterol-side-chain-cleavage activity, cells were preincubated for 1 h and incubated for 1 h (phosphorylation of proteins) or ½h (pregnenolone production) without or with

lutropin/1-methyl-3-isobutylxanthine. Labelled phosphate was present during the whole 60 min incubation period.

Homogenization of $(10–15) \times 10^6$ cells in 1 ml of 0.25 M-sucrose/1 mM-EDTA, pH 7.3, with or without 20 mM-NaF for labelling and pregnenolone incubations respectively, was performed with a Dounce glass homogenizer (clearance 0.025–0.03 mm; ten strokes). Differential centrifugation resulted in isolation of three fractions: nuclei (10 min at 500 *g*), mitochondria (10 min at 15000 *g*) and post-mitochondrial-supernatant (15000 *g* supernatant). Nuclear pellets were washed twice with homogenization medium. These fractions were characterized by determination of the DNA content and the cholesterol-side-chain cleavage, monoamine oxidase and lactate dehydrogenase activities in each fraction as described previously (Van der Vusse *et al.*, 1974).

Pregnenolone production was determined by incubation of subcellular fractions (0.015–0.300 mg of protein/500 μl) for 30 min in the presence of inhibitors (Van der Vusse *et al.*, 1974), but without calcium in the incubation medium. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Phosphorylated proteins were collected in the same way as described above for cellular proteins, except that postmitochondrial-supernatant proteins were precipitated with trichloroacetic acid [final concn. 10% (w/v)] before addition of SDS-containing sample medium. The subcellular localization of the lutropin-dependent phosphoproteins was determined by comparison of the distributions of marker enzymes and lutropin-dependent phosphoproteins. For this purpose the relative specific activities of both marker enzymes and phosphoproteins were calculated in the three fractions. Calculations of relative specific activity for phosphoproteins were based on densitogram tracings of autoradiograms obtained after electrophoresis, in two ways: (1) equal amounts of the protein-bound radioactivity in the three fractions were applied to the gel; (2) equal portions (e.g. one-third) of the total fractions were applied to the gel. Densitograms were obtained by scanning of the various slots taken from the autoradiograms. A blank section of the film was used to set the baseline and the peak heights of the lutropin-dependent phosphoproteins were measured in the different fractions. The relative-specific-activity values were calculated by dividing the percentage of a lutropin-dependent phosphoprotein (based on peak height) in a fraction by the percentage of protein in that fraction. When necessary, corrections were made for the portion of the fraction that was used for electrophoresis.

The two methods for determination of the relative-specific-activity values ($n = 3$, equal amounts of protein-bound radioactivity; $n = 2$, equal portions; n = number of different cell preparations) resulted in similar distributions of the phosphoproteins. The total amounts of protein-bound radioactivity in the various fractions were determined to calculate relative-specific-activity values for the incorporation of [^{32}P]orthophosphate into proteins by dividing the percentage of protein-bound radioactivity in a fraction by the percentage of protein in that fraction.

Results

The pregnenolone production in tumour Leydig cells was stimulated within 5 min after the addition of lutropin/1-methyl-3-isobutylxanthine (Fig. 1). The kinetics of lutropin-dependent phosphorylation of tumour-Leydig-cell proteins can be derived from the results presented in Fig. 2. Addition of lutropin/1-methyl-3-isobutylxanthine resulted also within 5 min in phosphorylation of proteins of 17000, 22000, 24000, 33000 and 57000 Da. The intensity of the lutropin-dependent phosphoproteins and, for

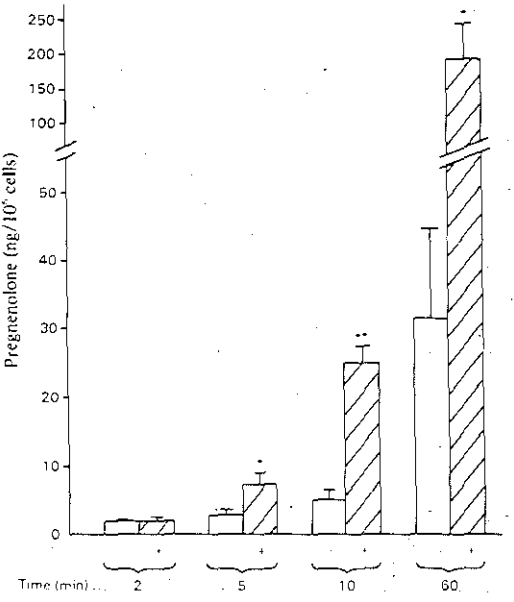

Fig. 1. *Pregnenolone productions in tumour Leydig cells in the absence (–) or presence (+) of lutropin/1-methyl-3-isobutylxanthine*

Incubations were performed as described in the text. Values are means ± S.D. for duplicate incubations of three different cell preparations. The significance of the lutropin-dependent increased pregnenolone production was determined by Student's paired *t* test with the corresponding incubations without lutropin/1-methyl-3-isobutylxanthine: $^*P < 0.025$; $^{**}P < 0.005$.

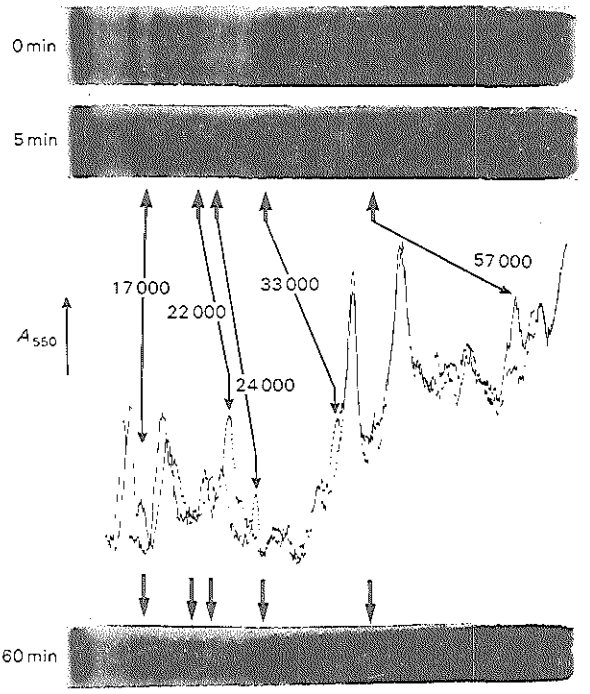

Fig. 2. *Kinetics of lutropin-dependent incorporation of [^{32}P]orthophosphate into proteins of tumour Leydig cells*
The Figure shows autoradiograms of [^{32}P]orthophosphate-labelled proteins isolated from tumour Leydig cells incubated for 0, 5 and 60 min with lutropin/1-methyl-3-isobutylxanthine (see the text) and separated by SDS/polyacrylamide-gel electrophoresis. Included above are densitograms obtained with the 0 and 5 min incubations. Arrows indicate lutropin-dependent phosphoproteins (numerical values are molecular masses in daltons).

Table 1. *Kinetics of lutropin-dependent incorporation of [^{32}P]orthophosphate into proteins of tumour Leydig cells*
Cells were incubated for 1 h in the presence of [^{32}P]orthophosphate, and lutropin/1-methyl-3-isobutylxanthine was added for the times indicated before the end of the incubation. Peak heights of the indicated proteins measured from the densitograms were expressed as the percentage of the peak height of a standard protein of molecular mass 40000 Da. Statistical analysis of the lutropin-dependent increments was done by Student's paired *t* test with the 0-min incubation. Results are means ± S.D. ($n = 3$–4); $^{*}P < 0.05$; $^{**}P < 0.01$.

Protein molecular mass (Da)	Incubation-time with lutropin/1-methyl-3-isobutylxanthine (min) ...	Incorporation (% peak height)			
		0	5	10	60
17000		14.3 ± 1.5	34.0 ± 5.2*	32.0 ± 8.0*	56.5 ± 8.7**
22000		37.3 ± 3.5	52.7 ± 1.5**	52.7 ± 4.7*	50.5 ± 4.0*
24000		17.2 ± 6.6	26.7 ± 4.9*	28.0 ± 9.0*	28.8 ± 6.2*
33000		47.0 ± 4.7	60.7 ± 7.6*	66.0 ± 10.4*	65.2 ± 7.9**
57000		72.5 ± 5.2	93.3 ± 5.0**	95.0 ± 3.6*	90.0 ± 5.9**
35000		89.0 ± 1.8	91.3 ± 3.2	90.3 ± 0.6	89.8 ± 2.2
50000		78.0 ± 5.3	82.7 ± 9.6	86.0 ± 9.2	82.8 ± 7.4
73000		106.3 ± 3.8	113.0 ± 3.6	112.7 ± 2.9	106.5 ± 6.4

comparison, of three other lutropin-independent phosphoproteins of 35000, 50000 and 73000 Da was expressed relative to a lutropin-independent phosphoprotein of 40000 Da (Table 1). The results showed that phosphorylation of the 22000, 24000, 33000 and 57000 Da proteins was almost maximal after 5 min of incubation with lutropin/1-methyl-3-isobutylxanthine, whereas phosphorylation of the

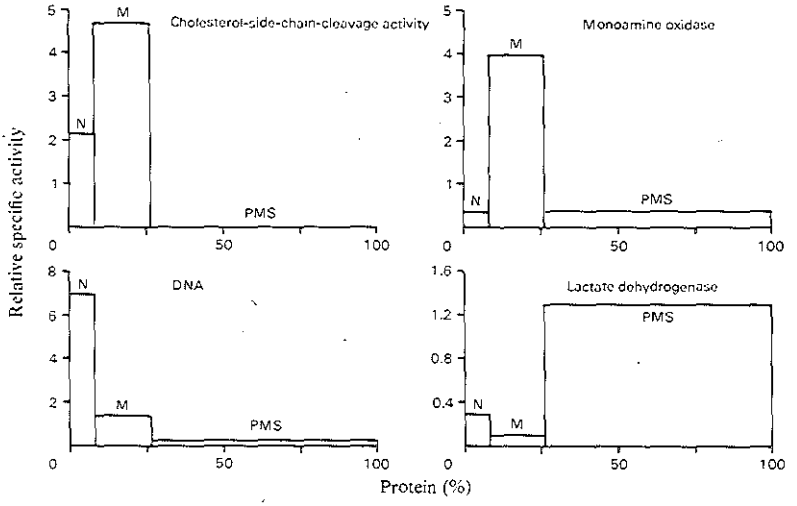

Fig. 3. *Characterization of subcellular fractions isolated from tumour Leydig cells*
Subcellular distribution of cholesterol-side-chain-cleavage activity, monoamine oxidase, DNA and lactate dehydrogenase is shown. Abscissae: the percentage of the total protein content in each fraction is presented as cumulative values. Ordinates: relative specific activity (percentage of total enzyme activity or amount of DNA per percentage of total protein content). Abbreviations used: N, nuclear fraction; M, mitochondrial fraction; PMS, post-mitochondrial-supernatant fraction. Results shown are mean values for three to five different cell preparations.

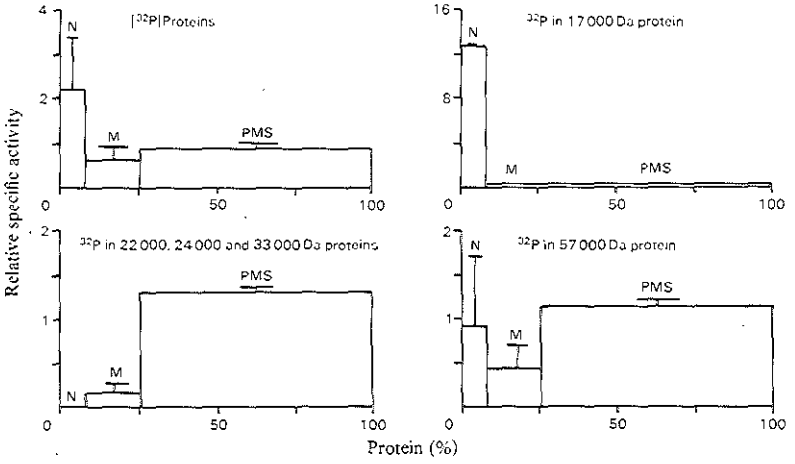

Fig. 4. *Subcellular distribution of ^{32}P incorporation into phosphoproteins and lutropin-dependent phosphoproteins in fractions of tumour Leydig cells*
Relative specific activities of the incorporation of [^{32}P]orthophosphate into proteins of subcellular fractions and relative-specific-activity values for the lutropin-dependent phosphoproteins, calculated as described in the text, are shown. Results are means ± S.D. for twelve (^{32}P incorporation) and three (phosphoproteins) different cell preparations. For details, see the Materials and methods section.

17000 Da protein increased during the whole 60 min incubation period. Finally, lutropin appeared to decrease the phosphorylation of a protein of 20000 Da.

The correlation between steroid production and phosphorylation of proteins was further investigated at the subcellular level. Three subcellular fractions were used: nuclei, mitochondria and postmito-

chondrial-supernatant. Particular attention was given to the mitochondrial fraction, because of the mitochondrial localization of the cholesterol-side-chain-cleavage activity (Simpson, 1979). The fractions were characterized by determining, in addition to the cholesterol-side-chain-cleavage activity, the DNA content and the activity of monoamine oxidase and lactate dehydrogenase.

The results showed that the nuclear fraction contained most of the DNA (55%) and that 96% of the lactate dehydrogenase activity was recovered in the postmitochondrial-supernatant fraction. Most of the activities of the mitochondrial marker enzymes (70–80%) were present in the mitochondrial fraction, but significant nuclear contamination and some postmitochondrial-supernatant activities were also present (Fig. 3).

Preincubation of cells with lutropin/1-methyl-3-isobutylxanthine for 30 min resulted in a 2.5-fold-increased pregnenolone production in the isolated mitochondria [194.6 ± 28.6 ng/30 min per mg of protein for the controls and 498.8 ± 91.5 ng/30 min per mg of protein for the mitochondria from lutropin/1-methyl-3-isobutylxanthine-stimulated cells (means $\pm$ S.D.: $n = 3$; $P < 0.01$)]. Relative specific activities were calculated for the incorporation of [^{32}P]orthophosphate into proteins of the fractions isolated (Fig. 4). In spite of the 4–5-fold purification of mitochondria in the mitochondrial fraction, the relative-specific-activity value of this fraction for ^{32}P incorporation into proteins was rather low [0.65 ± 0.35 (mean $\pm$ S.D., $n = 12$)].

The distribution of the separate lutropin-dependent phosphoproteins is also shown in Fig. 4. The 17000 Da protein could only be detected in the nuclear fraction, whereas the 22000, 24000, 33000 and 57000 Da proteins were concentrated in the postmitochondrial-supernatant fraction. A small amount of the 57000 Da protein was present in the nuclear and mitochondrial fraction. Qualitative analyses of the subcellular distribution of phosphoproteins showed that the phosphoproteins that had been concentrated in the nuclear and postmitochondrial-superantant fractions were present in the mitochondrial fraction (electrophoresis with equal amounts of protein-bound radioactivity) and that the mitochondrial fraction hardly contained phosphoproteins (electrophoresis with equal portions: Fig. 5).

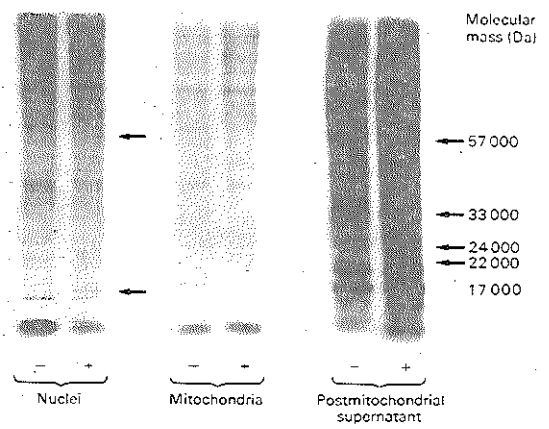

Fig. 5. *Subcellular distribution of phosphorylated proteins isolated from tumour Leydig cells incubated without* (−) *or with* (+) *lutropin/1-methyl-3-isobutylxanthine*

Autoradiograms of [^{32}P]orthophosphate-labelled proteins in subcellular fractions of tumour Leydig cells separated by SDS/polyacrylamide-gel electrophoresis are shown. Equal portions of the total amount of protein isolated in the fractions were applied.

Discussion

The aim of the present study was to investigate the kinetics of pregnenolone production and phosphorylation of proteins in tumour Leydig cells after addition of lutropin/1-methyl-3-isobutylxanthine. Both steroid production and phosphorylation of five proteins of 17000, 22000, 24000, 33000 and 57000 Da were significantly increased within 5 min after addition of lutropin/1-methyl-3-isobutylxanthine.

The possible role of the lutropin-dependent phosphoproteins was further investigated by determination of their subcellular localization. Characterization of subcellular fractions with marker enzymes showed that the distributions of the mitochondrial marker enzymes (cholesterol-side-chain-cleavage activity and monoamine oxidase) were similar and reflected an approx. 4-fold purification of mitochondria. Marker-enzyme activities for the nuclear and postmitochondrial-supernatant fractions were also present in the mitochondrial fraction. The incorporation of ^{32}P into proteins showed a low incorporation into proteins of the mitochondrial fraction. After electrophoresis of equal amounts of protein-bound radioactivity of subcellular fractions, the autoradiograms showed that the mitochondrial fraction contained mainly nuclear and postmitochondrial-supernatant phosphoproteins. Thus, in spite of the 4-fold purification of mitochondria, there appeared to be a low incorporation of [^{32}P]orthophosphate into proteins of mitochondria. Moreover, part of this activity originated from nuclear and postmitochondrial-supernatant contamination. On the basis of these observations it is concluded that activity of protein phosphorylation

in mitochondria is low. This conclusion is supported by the results in Fig. 5, and so far there have been reports on just a few mitochondrial proteins that become phosphorylated. Among the latter, succinyl-CoA synthetase and pyruvate dehydrogenase are the only phosphoproteins that have been identified (Weller, 1979). In fact, none of the lutropin-dependent phosphoproteins could be demonstrated in the mitochondrial fraction. The 17000 Da phosphoprotein was found in the nuclei and the 22000, 24000, 33000 and 57000 Da phosphoproteins were found in the postmitochondrial-supernatant fraction.

The lutropin-dependent phosphoprotein of 57000 Da appears to be the regulatory subunit of the type-II cyclic AMP-dependent protein kinase (Cooke *et al.*, 1979*b*). A partially membrane-bound localization of this regulatory subunit (cf. Potter & Taylor, 1979) could explain the presence of this protein in all fractions isolated. Nothing is known about the nature of the phosphoproteins of 22000, 24000 and 33000 Da, although there have been reports on cyclic AMP-binding proteins of similar molecular mass that may have originated from the 57000 Da protein after proteolytic cleavage (Weber & Hilz, 1978, 1979; Wallace & Frazier, 1979; Jaynes *et al.*, 1980). The 17000 Da protein could be involved in lutropin-induced protein synthesis, both in adult Leydig cells (Cooke *et al.*, 1977; Janszen *et al.*, 1977) and in tumour Leydig cells (Janszen *et al.*, 1978).

Hormone-dependent phosphorylation of proteins has also been reported for other steroidogenic cell types, such as rat adrenocortical cells (Podesta *et al.*, 1979), porcine granulosa cells (Halpren-Ruder *et al.*, 1980) and bovine luteal cells (Darbon *et al.*, 1980). In those studies a large number of phosphoproteins with molecular masses ranging from 43000 to 150000 Da was observed in cytosol fractions, but none of these phosphoproteins were functionally characterized. More recently, increased phosphorylation of phosphoproteins of 22000, 24000, 54000 and 210000 Da was demonstrated in rat adrenal quarters under the influence of corticotropin (Koroscil & Gallant, 1980). The 210000 Da phosphoprotein was reported to be localized in the mitochondrial fraction, but the characterization of the mitochondrial fraction was not supported by the distribution of proper marker enzymes. The rather low incorporation of labelled phosphate into mitochondrial proteins demonstrated in the present study may rule out involvement of phosphoproteins with enzymic processes occurring inside mitochondria. Studies with reconstituted cholesterol-side-chain-cleavage enzymes of bovine corpus luteum (Caron *et al.*, 1975) and bovine adrenal (Defaye *et al.*, 1981) gave conflicting results with regard to phosphorylation of the enzyme. The mitochondrial membranes may even prevent the entry of phosphoproteins into mitochondria. However, recent observations on the possible importance of polyphosphoinositides for stimulation of cholesterol-side-chain-cleavage activity in the adrenal (Farese *et al.*, 1979, 1980; Farese & Sabir, 1980) may suggest some role for hormone-dependent phosphoproteins in the post-mitochondrial-supernatant or cytosol. These phosphoproteins might stimulate synthesis of (poly)-phosphoinositides at the endoplasmic reticulum or facilitate in some way their transport to the mitochondria, where increased amounts of mitochondrial phosphoinositides could be involved in stimulating steroid production. It remains to be demonstrated to what extent the postmitochondrial-supernatant proteins demonstrated in the present study with tumour Leydig cells are involved in such a process.

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Appendix Paper II

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Lutropin increases phosphorylation of a 33 000-dalton ribosomal protein in rat tumour Leydig cells

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Addition of lutropin (luteinizing hormone, 'LH') and 3-isobutyl-1-methylxanthine to tumour Leydig cells stimulated phosphorylation of five proteins, of 17000, 22000, 24000, 33000 and 57000 Da. Phosphorylation of these proteins coincided with increased pregnenolone production. Phosphorylation of a 33000-Da protein was lutropin-dependent in Leydig cells isolated from a Leydig-cell tumour, from immature testes or from mature testes. In tumour Leydig cells this protein was present in the small ribosomal subunit. Incubation of tumour Leydig cells with either cycloheximide or puromycin inhibited both basal and lutropin-dependent pregnenolone production, by approx. 90% and 98% respectively. In contrast, basal pregnenolone production in Leydig cells from immature and mature testes was insensitive to cycloheximide or puromycin. Cycloheximide or puromycin increased phosphorylation of the 33000-Da phosphoprotein by approx. 130% and 80% respectively (effect of lutropin/3-isobutyl-1-methylxanthine on phosphorylation: 100%). The molecular mass, the subcellular localization and the sensitivity to phosphorylation in the presence of inhibitors of protein synthesis indicate that the 33000-Da protein could be similar to ribosomal protein S6.

The action of lutropin (luteinizing hormone, 'LH') on steroidogenesis in rat tumour Leydig cells is accompanied by activation of protein kinase and phosphorylation of proteins, and increased synthesis of specific proteins (Janszen *et al.*, 1978; Cooke *et al.*, 1979; Bakker *et al.*, 1981; for a review, see Rommerts & Brinkmann, 1981). It is not known whether, and if so how, phosphorylated proteins or newly synthesized proteins are related to the rate-limiting step in steroid production, i.e. the cholesterol-side-chain-cleavage activity in mitochondria. Lutropin-dependent phosphorylation of five proteins (17000, 22000, 24000, 33000 and 57000 Da) has been demonstrated in rat tumour Leydig cells, and phosphorylation of these proteins coincided with increased steroid production (Bakker *et al.*, 1981). The 17000-Da protein was isolated from the nuclear fraction, and the other four proteins were isolated from the postmitochondrial supernatant. None of the lutropin-dependent phosphoproteins was localized in mitochondria. Hence a direct effect of these phosphoproteins on the cholesterol-side-chain-cleavage enzyme appears unlikely.

Hormone [corticotropin (ACTH) or lutropin]-stimulated steroid production is dependent on protein synthesis (Schulster *et al.*, 1974; Cooke *et al.*, 1975; Farese *et al.*, 1980). Lutropin-dependent phosphoproteins present in the postmitochondrial supernatant may be involved in regulation of protein synthesis essential for steroidogenesis. In the present study we have therefore further investigated the localization and properties of the phosphoproteins present in the postmitochondrial supernatant, with particular reference to the ribosomes.

Materials and methods

Details of chemicals and methods used for isolation and incubation of tumour Leydig cells have been described previously (Bakker *et al.*, 1981). [^{32}P]P_i (carrier-free) and U-^{14}C-labelled L-amino acids mixture (100–500 Ci/mol) were purchased from New England Nuclear, Boston, MA, U.S.A.

Isolation of testis Leydig cells from immature (21–23 days) and mature (90–100 days) Wistar rats (substrain R_1-Amsterdam) was performed as described by Janszen *et al.* (1976), except that no dextran-

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centrifugation step was used. Incubation of cells was essentially as described by Bakker *et al.* (1981). In brief, cells were preincubated for 60 min and incubated with or without lutropin/3-isobutyl-1-methylxanthine as indicated (lutropin concentrations: 100 ng/ml for immature and mature testis Leydig cells, 1000 ng/ml for tumour Leydig cells; 3-isobutyl-1-methylxanthine: 0.25 mM). Incubations were performed in the presence of inhibitors of pregnenolone metabolism or [^{32}P]P_i, and with or without cycloheximide (89 μM) or puromycin (120 μM).

For isolation of ribosomes, tumour Leydig cells were incubated at 32°C under an atmosphere of 5% CO_2 in air in Krebs–Ringer buffer without phosphate (pH 7.3), with 0.2% glucose and 0.1% albumin (4×10^7 cells/2.5 ml). Labelling was performed for 60 min with 125 μCi of [^{32}P]P_i in the absence or presence of lutropin/3-isobutyl-1-methylxanthine or cycloheximide, as indicated. Subsequently, cells were transferred to tubes containing 5 ml of 0.125 M-NaH_2PO_4/20 mM-NaF/82 mM-Tris (pH 7.3). Ribosomes were isolated as described by Walton & Gill (1973). Cells were centrifuged (10 min at 100 *g*), resuspended and homogenized in 0.25 M-sucrose/50 mM-Tris/HCl (pH 7.5)/25 mM-KCl/5 mM-$MgCl_2$/20 mM-NaF, with a Dounce glass homogenizer as described by Bakker *et al.* (1981). After removal of cell debris (10 min at 100 *g*) and nuclei plus mitochondria (10 min at 15000 *g*), the isolated post-mitochondrial supernatant was adjusted to 4% Triton X-100 and 1% sodium deoxycholate. A ribosomal pellet was obtained by centrifugation in a Beckman SW 40 rotor for 2 h at 29000 rev./min (105000 $g_{av.}$). Ribosomes were resuspended in 150 μl of TMD buffer [2 mM-Tris/

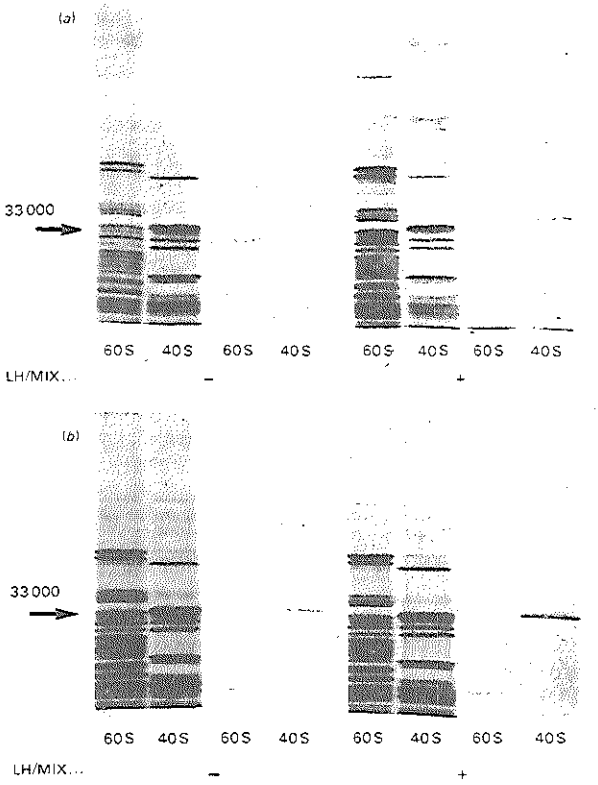

Fig. 1. *Subcellular localization of the lutropin-dependent phosphoprotein of 33000 Da and the effect of cycloheximide on its phosphorylation*

Tumour Leydig cells were incubated for 60 min with [^{32}P]P_i in the absence (–) or presence (+) of lutropin/3-isobutyl-1-methylxanthine (LH/MIX): (*a*) without cycloheximide; (*b*) with cycloheximide. Ribosomes were isolated (see the Materials and methods section) and proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Each panel shows the protein staining (on the left) and the corresponding radioautogram (on the right).

HCl (pH 7.5)/0.5 mM-$MgCl_2$/0.1 mM-dithiothreitol]. The suspension was adjusted to 250 mM-KCl by addition of 50 μl of 1 M-KCl in TMD buffer, and layered on top of a linear 15–35% sucrose gradient (11 ml) in 10 mM-Tris/HCl (pH 7.5)/250 mM-KCl/2 mM-$MgCl_2$. The gradients were centrifuged in a Beckman SW 40 rotor for 15 h at 25500 rev./min (80900 $g_{av.}$). The A_{260} pattern of the gradient was recorded. Fractions of the gradient containing the 40S and 60S subunits were pooled and adjusted to 10 mM-$MgCl_2$. Ribosomal proteins were precipitated by the addition of 0.7 vol. of ethanol. Finally, proteins were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Bakker *et al.*, 1981).

For determination of incorporation of ^{14}C-labelled amino acids into proteins, cells were incubated in Minimal Essential Medium (Grand Island Biological Co., Grand Island, NY, U.S.A.), containing (per litre): 1.19 g of L-glutamine, 100 mg of streptomycin, 100000 units of penicillin and 625 μg of Fungizone. Incubations were performed at 32°C under air/CO_2 (19:1) in medium containing 1% foetal-calf serum. During 60 min of preincubation, tumour Leydig cells attached to Petri dishes (approx. 2×10^6 cells/dish). Incubations for 30 min in the presence of 10 μCi of ^{14}C-labelled amino acids were performed in a final volume of approx. 850 μl with or without lutropin/3-isobutyl-1-methylxanthine. After incubation, attached cells were washed with 5 × 1 ml of 0.9% NaCl and then 5 × 1 ml of 10% trichloroacetic acid (all at 4°C). Finally, cells were dissolved in 1 ml of 1 M-NaOH for determination of radioactivity and protein content. For determination of incorporation of [^{32}P]P_i into proteins, an incubation similar to that described above was performed with cells attached to Petri dishes (approx. 2×10^6 cells/dish). Incubations were for 60 min with 125 μCi of [^{32}P]P_i in 2 ml of Krebs–Ringer buffer without phosphate (pH 7.3), containing 0.2% glucose and 1% foetal-calf serum.

Results

Ribosomes were isolated from tumour Leydig cells that had been incubated for 60 min with [^{32}P]P_i with or without lutropin/3-isobutyl-1-methylxanthine. After homogenization of the cells, the 40S and 60S ribosomal subunits were separated on 15–35% sucrose gradients. Phosphorylated proteins obtained from the ribosomal subunits were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The lutropin-dependent 33000-Da phosphoprotein was present in the 40S subunit (Fig. 1). Phosphorylation of a protein with the same molecular mass was stimulated by lutropin also in immature and mature testis Leydig cells (Fig. 2). Effects of inhibitors of protein synthesis on pregnenolone production and phosphorylation of proteins were investigated with concentrations of cycloheximide (89 μM) and puromycin (120 μM) as described by Cooke *et al.* (1975) for mature testis Leydig cells. These concentrations inhibited protein synthesis in all Leydig cells investigated to the same extent (approx. 95%). Cycloheximide or puromycin inhibited both basal and lutropin-dependent pregnenolone production in tumour Leydig cells by approx. 90% and 98% respectively (Table 1). In contrast, a specific phosphorylation of the 33000-Da protein was detected in the presence of cycloheximide (Fig. 3) or puromycin (results not shown). Phosphorylation was increased by 130% and 80% in the presence of cycloheximide and puromycin respectively [stimulation by lutropin/3-isobutyl-1-methylxanthine = 100% (Table 1: see also Fig. 1)]. Moreover, a synergism of lutropin and cycloheximide was observed with regard to phosphorylation of the 33000-Da protein (Table 1).

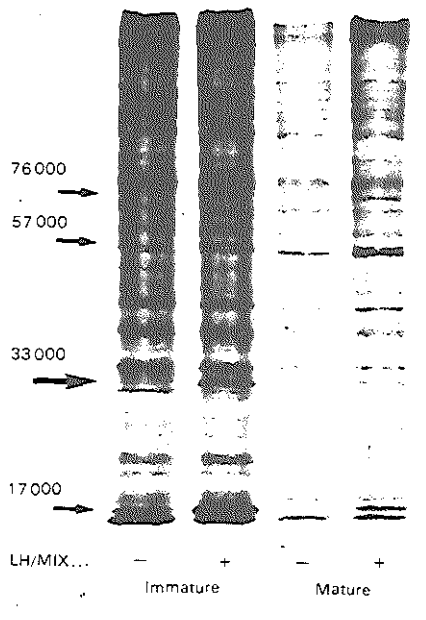

Fig. 2. *Radioautogram showing the lutropin-dependent phosphorylation of the 33000-Da protein in immature and mature testis Leydig cells*

Leydig cells were incubated for 60 min with [^{32}P]P_i in the absence (–) or presence (+) of lutropin/3-isobutyl-1-methylxanthine (LH/MIX). Phosphorylated proteins were isolated and separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Arrows indicate lutropin-dependent phosphoproteins and their molecular masses in daltons.

Table 1. *Effect of inhibitors of protein synthesis on pregnenolone production and incorporation of $[^{32}P]P_i$ into the 33000-Da phosphoprotein in tumour Leydig cells*

Cells were incubated for 60min with inhibitors of pregnenolone metabolism or $[^{32}P]P_i$ as described previously (Bakker *et al.*, 1981). Parallel incubations (in duplicate) were performed in the absence (–) or presence (+) of cycloheximide (CX), puromycin (PUR) or lutropin/3-isobutyl-1-methylxanthine (LH/MIX). Peak heights of the 33000-Da phosphoprotein measured from the densitograms were expressed as the percentage of the peak height of a standard protein of molecular mass 40000Da. Results are means ± S.D., for the numbers of different cell preparations shown in parentheses. Statistical analysis was done by Student's paired *t* test: n.s., not significant.

			Pregnenolone		Phosphate in 33000-Da protein		
LH/MIX	CX (89μM)	PUR (120μM)	(ng/10^6 cells)	Change versus '–LH/MIX'	(% of peak height)	Change versus '–LH/MIX'	Effect of inhibitor versus '+LH/MIX'
–	–	–	41.1 ± 9.1 (5)	—	69.3 ± 6.3 (4)	—	—
+	–	–	250.0 ± 31.1 (5)	$P < 0.005$	78.2 ± 3.7 (4)	$P < 0.025$	—
–	+	–	4.7 ± 1.6 (5)	$P < 0.005$	81.4 ± 7.1 (4)	$P < 0.005$	—
+	+	–	7.0 ± 1.7 (5)	$P < 0.005$	90.5 ± 4.3 (4)	$P < 0.005$	$P < 0.005$
–	–	+	4.2 ± 1.5 (5)	$P < 0.005$	76.3 ± 5.5 (3)	$P < 0.025$	—
+	–	+	5.1 ± 1.7 (5)	$P < 0.005$	82.3 ± 6.8 (3)	$P < 0.01$	n.s.

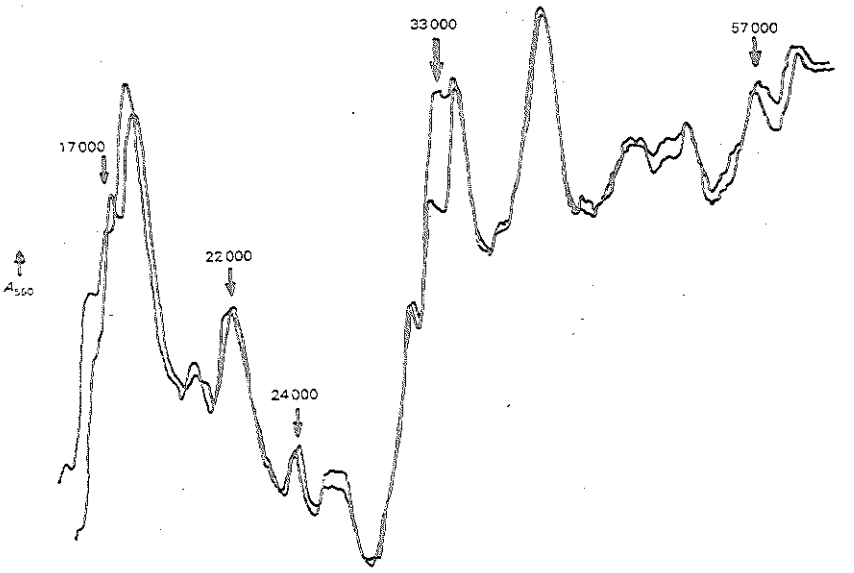

Fig. 3. *Effects of lutropin/3-isobutyl-1-methylxanthine and cycloheximide on phosphorylation of proteins of tumour Leydig cells*

Phosphorylated proteins were isolated from cells incubated in the presence of lutropin/3-isobutyl-1-methylxanthine with or without cycloheximide. Radioautograms obtained after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were scanned with a Gilford spectrophotometer (for full details see Bakker *et al.*, 1981). Arrows indicate lutropin-dependent phosphoproteins. The large arrow indicates the additional phosphorylation of the 33000-Da phosphoprotein observed in the presence of cycloheximide. Numerical values are molecular masses in daltons.

Kinetic studies of the inhibition of pregnenolone production and the phosphorylation of the 33000-Da protein were performed with cycloheximide. The decrease with time in basal and lutropin-stimulated pregnenolone production after addition of cycloheximide was more or less the same, which indicated the possible involvement of a protein(s) with a half-life of approx. 6min (Fig. 4). A gradually

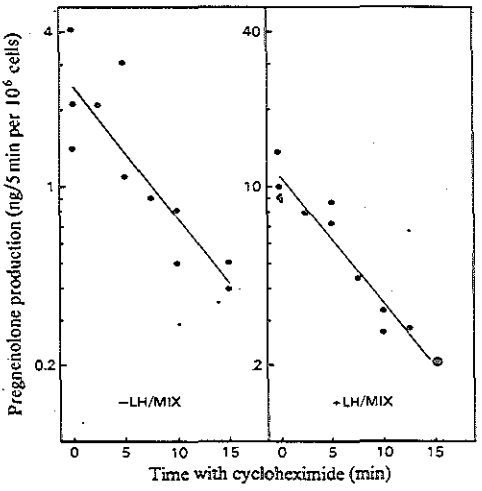

Fig. 4. *Kinetics of the effect of cycloheximide on pregnenolone production by tumour Leydig cells*
Cells were incubated for 30 min with or without lutropin/3-isobutyl-1-methylxanthine (LH/MIX). Subsequently, cycloheximide was added and pregnenolone production was determined during 5 min periods. Results shown are the mean values obtained in three experiments with duplicate incubations. Further data for the regression lines shown for the incubations without and with lutropin/3-isobutyl-1-methylxanthine respectively: number of mean values, 11 and 12; correlation coefficient, −0.85 and −0.96; half-time of the decrease, 5.8 min and 6.0 min.

increasing phosphorylation of the 33000-Da protein was observed during incubations for 60 min in the presence of cycloheximide and with or without lutropin/3-isobutyl-1-methylxanthine (results not shown). Unlike tumour Leydig cells, basal pregnenolone production in immature and mature testis Leydig cells was not inhibited by either cycloheximide (Table 2) or puromycin (results not shown).

Increased phosphorylation of the 33000-Da ribosomal protein might be the consequence of increased specific radioactivity of [γ-^{32}P]ATP caused by increased uptake of [^{32}P]P_i in the presence of lutropin/3-isobutyl-1-methylxanthine. On the other hand, phosphorylation of the 33000-Da protein may be involved in regulation of protein synthesis. Therefore incorporation of [^{32}P]P_i and ^{14}C-labelled amino acids into proteins was determined. However, no effects of lutropin/3-isobutyl-1-methylxanthine could be demonstrated (Table 3).

Table 2. *Pregnenolone production in Leydig cells isolated from immature testes, mature testes and a Leydig-cell tumour*
Leydig cells were incubated for 60 min in the absence (−) or presence (+) of lutropin/3-isobutyl-1-methylxanthine (LH/MIX) or cycloheximide (CX). Results are means ± S.D. for the numbers of different cell preparations shown in parentheses. Statistical analysis was done by Student's paired *t* test: n.s., not significant.

		Immature Leydig cells			Mature Leydig cells			Tumour Leydig cells		
LH/MIX	CX (89 μM)	(ng/10^6 cells)	Effect of LH/MIX	Effect of CX	(ng/10^6 cells)	Effect of LH/MIX	Effect of CX	(ng/10^6 cells)	Effect of LH/MIX	Effect of CX
−	−	0.5 ± 0.1 (3)	—	—	7.2 ± 1.1 (2)	—	—	41.1 ± 9.1 (5)	—	—
+	−	17.8 ± 4.3 (3)	$P < 0.025$	—	39.5 ± 3.5 (2)	$P < 0.025$	—	250.0 ± 31.1 (5)	$P < 0.005$	—
−	+	0.5 ± 0.1 (3)	—	n.s.	6.6 ± 1.5 (2)	—	n.s.	4.7 ± 1.6 (5)	—	$P < 0.005$
+	+	0.7 ± 0.1 (3)	$P < 0.05$	$P < 0.025$	6.6 ± 0.6 (2)	n.s.	$P < 0.025$	7.0 ± 1.7 (5)	$P < 0.005$	$P < 0.005$

Table 3. *Incorporation of [^{32}P]P_i and ^{14}C-labelled amino acids into proteins of tumour Leydig cells*
Cells were incubated in the absence (−) or presence (+) of lutropin/3-isobutyl-1-methylxanthine with either 125 μCi of [^{32}P]P_i or 10 μCi of ^{14}C-labelled amino acids. Incorporation of label was determined as described in the Materials and methods section. Results shown are values obtained with duplicate incubations (mean ± range).

Lutropin/3-isobutyl-1-methylxanthine	Expt. 1 (10^{-3} × ^{32}P c.p.m./60 min per mg)	Expt. 2 (10^{-3} × ^{14}C d.p.m./30 min per mg)	Expt. 3 (10^{-3} × ^{14}C d.p.m./30 min per mg)
−	587.0 ± 57.1	101.4 ± 5.4	165.1 ± 3.5
+	523.1 ± 34.2	101.8 ± 10.8	162.4 ± 6.5

Discussion

The aim of the present study was to investigate further the localization of lutropin-dependent phosphoproteins demonstrated in tumour Leydig cells (Bakker *et al.*, 1981), which might be involved in regulation of steroidogenesis. The results indicate that a lutropin-dependent phosphoprotein of 33000 Da was present in the 40S ribosomal subunit isolated from tumour Leydig cells. Cycloheximide and puromycin also stimulated phosphorylation of this protein, under conditions in which basal and lutropin-dependent pregnenolone productions were inhibited. The decrease with time in pregnenolone production under basal and stimulated conditions was about the same, which suggests the possible involvement of a protein(s) with a short half-life (approx. 6 min).

Unlike tumour Leydig cells, basal pregnenolone production in immature and mature testis Leydig cells was not inhibited by cycloheximide (or puromycin). Basal pregnenolone production expressed as ng/60 min per 10^6 cells in tumour Leydig cells is relatively high (approx. 80 times and 6–7 times the basal pregnenolone production in immature and mature testis Leydig cells respectively). Apart from different contents of Leydig cells in the various preparations (cf. Janszen *et al.*, 1976; Cooke *et al.*, 1979), the higher basal pregnenolone production in tumour Leydig cells may be due to increased basal amounts of cyclic AMP (cf. Cooke *et al.*, 1976, 1979). This would explain the comparable kinetics of inhibition by cycloheximide of pregnenolone production under basal and stimulated conditions (Fig. 4).

Effects of protein-synthesis inhibitors on phosphorylation of ribosomal protein S6 have been reported (Gressner & Wool, 1974; Lastick & McConkey, 1980). The molecular mass, the subcellular localization and the sensitivity to phosphorylation in the presence of inhibitors of protein synthesis suggest that the 33000-Da protein could be similar to protein S6. Immature and mature testis Leydig cells also showed a lutropin-dependent phosphoprotein of 33000-Da, which suggests that in Leydig cells from rat testis lutropin-dependent phosphorylation of ribosomal protein S6 occurs.

The increased phosphorylation of ribosomal protein S6 in the presence of inhibitors of protein synthesis has been explained by inhibition of metabolism of cyclic nucleotides (Gressner & Wool, 1974; Leader, 1980). However, increased amounts of cyclic AMP will probably stimulate cyclic AMP-dependent protein kinase in a non-specific way, resulting in increased phosphorylation of all cyclic AMP-dependent phosphoproteins. In the present study we observed an effect only on phosphorylation of protein S6. Therefore, increased phosphorylation of a specific protein in the presence of cycloheximide or puromycin cannot be easily explained by a non-specific increase in cyclic AMP. Moreover, since no effect of lutropin/3-isobutyl-1-methylxanthine could be detected on the incorporation of [^{32}P]P_i into proteins phosphorylation owing to changes in the specific radioactivity of [γ-^{32}P]ATP is unlikely. The increase in phosphorylation of ribosomal protein S6 was higher in the presence of cycloheximide than of puromycin, which may be related to the different actions of cycloheximide and puromycin. The action of cycloheximide (inhibition of the peptidyl-translocation step of elongation) involves binding to the ribosome, resulting in inhibition of passage of the ribosome along the mRNA. The action of puromycin, however, involves binding to the growing peptide chain, which causes release of this peptide chain and dissociation of the ribosomal subunits. Hence, the greater stimulation of phosphorylation of ribosomal protein S6 in the presence of cycloheximide shown in our study and in other studies (Gressner & Wool, 1974; Lastick & McConkey, 1980) might be due to steric hindrance at the ribosome. Phosphorylation of protein S6 in the presence of cycloheximide increased with time, which may indicate that its phosphorylation continues under the influence of the basal activity of probably cyclic AMP-dependent protein kinase (see above), whereas dephosphorylation is impaired or even impossible, owing to the presence of cycloheximide. During incubations of ribosomes isolated from bovine adrenal (Walton & Gill, 1973) and corpus luteum (Azhar & Menon, 1975) with cyclic AMP-dependent protein kinase, phosphorylation of a single protein in the 40S

subunit and of six to twelve proteins in the 60S subunit has been observed. The greater number of phophoproteins in those studies may represent an artifact *in vitro* (see, e.g., Krebs & Beavo, 1979).

Phosphorylation of ribosomal protein S6 has been frequently related to increased protein synthesis (Leader, 1980). However, in tumour Leydig cells incorporation of amino acids into proteins was not stimulated by lutropin/3-isobutyl-1-methylxanthine during 30min of incubation, whereas lutropin-dependent phosphorylation of protein S6 was almost maximal after 5min of incubation (Bakker *et al.*, 1981). It is possible, however, that synthesis of some specific protein, i.e. the rapidly-turning-over protein(s) required for hormone-dependent steroid production, is increased via phosphorylation of proteins involved in protein synthesis.

It is known that cyclic AMP can stimulate phosphorylation of protein S6 in some other cell types: hepatocytes (Gressner & Wool, 1976), thymocytes (Wettenhall & Howlett, 1979), HeLa cells (Lastick & McConkey, 1980). Moreover, cyclic AMP can stimulate synthesis of specific proteins such as tyrosine aminotransferase (Wicks *et al.*, 1969; Snoek *et al.*, 1981) and phosphoenolpyruvate carboxykinase (Iynedjian & Hanson, 1977) in rat hepatocytes and hepatoma cells, with no detectable increase in general protein synthesis. The increased synthesis of tyrosine aminotransferase in rat hepatoma cells in response to cyclic AMP appears to reflect an increase in the rate of initiation on the tyrosine aminotransferase mRNA (Snoek *et al.*, 1981). In Leydig cells a similar mechanism could operate for the induction of the rapidly-turning-over protein(s) required for lutropin-dependent steroid production. The lutropin-dependent phosphorylation of ribosomal protein S6 and proteins of 22000 and 24000 Da (Bakker *et al.*, 1981) might participate in this mechanism of specific translation.

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Appendix Paper III

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LH-DEPENDENT STEROID PRODUCTION AND PROTEIN PHOSPHORYLATION DURING SHORT-TERM CULTURE OF RAT TUMOUR LEYDIG CELLS

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SYNOPSIS

The correlation between changes in hormone-dependent pregnenolone production and phosphorylation of six proteins has been investigated with tumour Leydig cells cultured at 32°C or 37°C for two days in order to establish which phosphoprotein may play a predominant role in the regulation of steroid production.

Pregnenolone production and phosphorylation of proteins of 17000, 22000, 24000, 33000 and 57000 Da which were stimulated after addition of lutropin/1-methyl-3-isobutylxanthine (LH/MIX; 1000 ng/ml and 0.25 mM resp.) or dibutyryl cyclic AMP (dbcAMP; 0.5 mM) declined after a two days culture period to approx. 15% and 40% of the original activities. A 20000 Da phosphoprotein was dephosphorylated under the influence of LH/MIX or dbcAMP, and this hormone-dependent dephosphorylation also decreased during culture.

LH-independent protein phosphorylation was not changed even after five days of culture at 32°C. Incubation of cells at 37°C rather than 32°C significantly stimulated pregnenolone production on day 0, whereas on day 1 cells were less active at 37°C than at 32°C.

The capacity of the cholesterol side-chain cleavage (CSCC) activity (tested with 25-hydroxycholesterol; 32 μM) which was at least seven-fold greater than the maximal LH/MIX-stimulated activity, was on day 2 approx. 44% of the activity on day 0.

These results indicate that in isolated cells in culture a defect in the steroidogenic pathway develops between cyclic AMP (cAMP) and CSCC. This defect may be at the level of the hormone-dependent protein phosphorylation because phosphorylation of all the 6 phosphoproteins studied decreased to the same extent. The change in LH-stimulated phosphorylation of the 57000 Da protein (the regulatory subunit of the type II cyclic AMP-dependent protein kinase) during culture closely correlated with the decline in activity of control and cAMP-stimulated protein kinase during culture of cells at 32°C and at 37°C. Thus, the decreased activity of cAMP-dependent protein kinase may account for the observed changes in phosphorylation of LH-dependent phosphoproteins and will contribute to the decreased steroid production.

Abbreviations: LH, luteinizing hormone, lutropin;
MIX, 1-methyl-3-isobutylxanthine;
cAMP, cyclic adenosine-3':5'-monophosphate;
dbcAMP, N^6-2'-O-dibutyryl cyclic adenosine-3':5'-monophosphate;
ACTH, adrenocorticotropic hormone;
CSCC, cholesterol side-chain cleavage;

Trivial name: 25-hydroxycholesterol, 5-cholestene-3β,25-diol

Running title: LH-responsiveness of cultured tumour Leydig cells

Indexing words: Leydig cell culture/protein phosphorylation
steroidogenesis

INTRODUCTION

Phosphoproteins play a very important role in the regulation of cellular metabolism (Cohen, 1982). Hormones can regulate the phosphorylation of these phosphoproteins via three different protein kinase systems (Nishizuka et al., 1979). Studies with adrenal cell mutants containing reduced activities of adenylate cyclase and cAMP-dependent protein kinase have shown that the cAMP-dependent protein kinase system is essential for hormone-stimulated steroid production (Rae et al., 1979, 1980; cf. also: Lin et al., 1982).

Hormonal stimulation of steroidogenic cells is accompanied by an increase in the cAMP concentration (reviews by Marsh, 1976; Schimmer, 1980), activation of cAMP-dependent protein kinase (Cooke et al., 1976; Podesta et al., 1976; review by Schimmer, 1980) and increased phosphorylation of proteins (Cooke et al, 1977; Podesta et al., 1979; Koroscil & Gallant, 1980; Bakker et al., 1981). The similarities in the kinetics of increased steroid production and phosphorylation of specific proteins after addition of ACTH or LH suggest that phosphoproteins could be essential for regulation of steroid production (Koroscil & Gallant, 1980; Bakker et al., 1981; Darbon et al., 1981).

We have recently demonstrated that phosphorylation of five proteins of 17000, 22000, 24000, 33000 and 57000 Da and pregnenolone production in tumour Leydig cells are increased within 5 min after addition of LH/MIX (Bakker et al., 1981). Hence, all these LH-dependent phosphoproteins could be essential for acute regulation of steroid production by LH. However, the rapid phosphorylation of the five LH-dependent phosphoproteins made it impossible to assess with kinetic experiments the importance of individual phosphoproteins. Recently, several studies have reported a decrease in stimulated steroid production of Leydig cells in culture (Hsueh, 1980; Hunter et al. 1982; Murphy & Moger, 1982; Rommerts et al., 1982; Verhoeven et al., 1982). Preliminary results with tumour Leydig cells in culture also showed a decreae in LH-stimulated formation of phosphoproteins. Hence, experiments were carried out to investigate which phosphoproteins decreased in parallel with steroid production. Such phosphoproteins might be important for the regulation of the CSCC activity, and might be involved in the decreased responsiveness of cultured Leydig cells.

MATERIALS AND METHODS

Chemicals and methods used for isolation and incubation of tumour Leydig cells were essentially as described previously (Bakker et al., 1981; 1982).

(γ-^{32}P)adenosine-5'-triphosphate (1000-3000 Ci/mmol; γ-^{32}P-ATP) was purchased from New England Nuclear, Boston, Mass., U.S.A. Cyclic adenosine-3':5'-monophosphate (cAMP), N^6-2'-O-dibutyryl cyclic AMP (dbcAMP) and adenosine-5'-triphosphate (ATP) were from Boehringer, Mannheim, Germany. 5-Cholestene-3β,25-diol (25-hydroxycholesterol) was from Steraloids Inc., Wilton, NH, U.S.A.

Isolation and incubation of tumour Leydig cells

Tumour Leydig cells were obtained by dispersion of tumour fragments (Bakker et al., 1981) with collagenase containing 0.1% albumin for 25 min at 37°C. Isolated Leydig cells were immediately incubated in Petri dishes (1-2 x 10^6 cells per dish; 35 x 10 mm) with 2 ml Minimum Essential Medium (Grand Island Biol. Comp., Grand Island, NY, U.S.A.), containing per litre: 1.19 g L-glutamine, 100 mg streptomycin, 100,000 I.U. penicillin and 625 µg Fungizone.

Incubations were performed at 32°C or 37°C under an air/CO_2 (95 : 5) atmosphere in medium containing 1% foetal calf serum. During the first hour of incubation, Leydig cells attached to the Petri dishes. Attached cells were washed three times with Krebs-Ringer buffer without phosphate (pH 7.3) containing 0.2% glucose and 0.1% albumin, immediately before the estimation of pregnenolone production or protein phosphorylation during the 2nd (day 0), 20th (day 1) or 44th hour (day 2) of incubation. Final concentrations of chemicals added to the Leydig cells in this medium were: LH, 1000 ng/ml; MIX, added with LH, 0.25 mM; dbcAMP, 0.5 mM; 25-hydroxycholesterol, 32 µM.

Estimation of pregnenolone production

Pregnenolone production was determined with 1 x 10^6 cells in a final volume of approx. 2 ml during a 60 min incubation period in the presence of inhibitors of pregnenolone metabolism (Bakker et al., 1981). After incubation the medium was extracted two times with 2 ml ethyl acetate, and the amount of pregnenolone was determined by radioimmunoassay. Attached cells were washed with 0.15 M NaCl and dissolved in 1 ml 1 M NaOH for determination of the amount of protein.

Phosphorylation of proteins

Phosphorylation of proteins was performed with 2 x 10^6 cells in a final volume of approx. 600 µl, incubated for 60 min with 100 µCi (^{32}P)P_i. After incubation, cells were washed with cold (0-4°C) 0.125 M NaH_2PO_4, 82 mM Tris/HCl and 20 mM NaF (pH 7.3). Cells were dissolved in hot (80-90°C) sodium dodecyl sulphate (SDS)-containing lysing buffer and phosphorylated proteins were isolated, separated by SDS-polyacrylamide gel electrophoresis and quantified as described previously (Bakker et al., 1981).

Estimation of activity of cAMP-dependent protein kinase

Activity of cAMP-dependent protein kinase was estimated essentially as described by Corbin et al. (1973). Buffer solutions used were: Buffer A: 10 mM KH_2PO_4, 10 mM EDTA, 2 mM theophylline (pH 6.5); Buffer B: 17 mM KH_2PO_4, 6 mM magnesium acetate (pH 6.8). Additional solutions made with Buffer B were: 400 mM NaF; 6 µM cAMP; 10 mg/ml histones (Worthington Biochem. Corp., Freehold, NJ, U.S.A.), containing 2 µg/ml ATP.

Attached cells (2 x 10^6 per dish) were washed with Krebs-Ringer buffer without phosphate as described above and were subsequently detached from the dishes with 1.2 ml Buffer A, containing 0.1% Triton X-100. Lysis of cells was performed by sonication for 5 sec at 0°C. Protein kinase activity was assayed in triplicate in a final volume of 100 µl. 5 µl 400 mM NaF, 10 µl Buffer B (= without cAMP) or 10 µl 6 µM cAMP, 15 µl (γ-^{32}P)ATP (approx. 0.2 µCi; in Buffer B), 50 µl histones/ATP solution were mixed and kept on ice. After the addition of 20 µl enzyme solution in Buffer A (approx. 6 µg protein) incubation was carried out for 10 min at 37°C. Incubation was stopped by the 1 : 1 (by vol.) addition of 20% trichloroacetic acid (TCA). Precipitated histones were dissolved in 200 µl SDS-containing lysing buffer (Bakker et al., 1981) together with 10-20 µl 1 M Tris. Washing of phosphorylated histones was performed by precipitation with 20% TCA and redissolution in SDS-containing lysing buffer (+ Tris) (two times). Histones dissolved in SDS-containing lysing buffer were assayed for ^{32}P-radioactivity and the amount of protein (Peterson, 1977).

Blank incubations (i.e. without the addition of enzyme solution) contained less than 0.2% of the added amount of (γ-^{32}P)ATP, which was less than 14% of the amount of radioactivity incorporated into histones in control incubations. The coefficient of variation of triplicate estimations of protein kinase activity was 8.3% $\pm$ 5.2 (mean $\pm$ S.D.; n = 26). Incorporation of ^{32}P into histones in the presence of cAMP was 4-6 fold higher than in control incubations.

RESULTS

Characterization of cell viability

Isolated tumour Leydig cells could be maintained in culture for more than seven days without morphological signs of degeneration. A slight increase in protein content (at 32°C on day 2 versus day 0: 18% ± 18, $p < 0.005$, $n = 24$; at 37°C on day 1 versus day 0: 15% ± 3, $p < 0.005$, $n = 24$) and incorporation of ^{14}C-labelled amino acids in proteins (at 32 °C on day 2 versus day 0: 21% ± 16, not significant, $n = 6$; at 37°C on day 1 versus day 0: 47% ± 25, $p < 0.005$, $n = 6$) during culture indicated that the cells also remained functionally viable. The electrophoretic patterns of LH-independent protein phosphorylation on day 0 and day 5 were similar (Fig. 1).

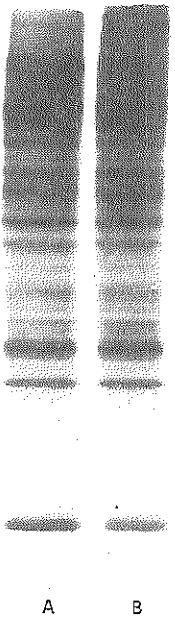

Fig. 1. Effect of culture on LH-independent protein phosphorylation. Tumour Leydig cells were cultured for 1 h (A) or 120 h (B) and subsequently incubated for 60 min with (^{32}P)P_i. Phosphorylated proteins were isolated and separated by SDS-polyacrylamide gel electrophoresis. The Figure shows the autoradiogram obtained.

Pregnenolone production

Pregnenolone production by tumour Leydig cells incubated at 32°C was determined on day 0, day 1 and day 2 of culture in the presence of LH/MIX, dbcAMP or 25-hydroxycholesterol. Stimulated pregnenolone production (i.e. control production subtracted) decreased with time under all conditions tested (Table 1). Basal pregnenolone production decreased from approx. 45 ng on day 0 and 1 to approx. 15 ng/60 min/mg protein on day 2.

Pregnenolone production stimulated with LH/MIX or dbcAMP on day 1 and day 2 was approx. 75% and 17% of the activity on day 0 (Table 1). DbcAMP-stimulated pregnenolone production was always significantly higher ($p < 0.05$) than the LH/MIX-stimulated pregnenolone production. The CSCC activity estimated with 25-hydroxycholesterol, which was always at least seven-fold higher than the LH/MIX-stimulated activity and at least four-fold higher than the dbcAMP-stimulated activity, was approx. 93% (on day 1) and approx. 44% (on day 2) of the activity on day 0.

Incubation of cells at 37°C instead of 32°C resulted in a significantly higher stimulated pregnenolone production on day 0, whereas pregnenolone production by cells incubated for 24 h at 37°C was significantly lower ($p < 0.005$; $n = 12$; Table 1).

Protein phosphorylation

Phosphorylation of proteins was investigated in the absence or presence of LH/MIX or dbcAMP. Both agents stimulated phosphorylation of five proteins of 17000, 22000, 24000, 33000 and 57000 Da and dephosphorylation of a protein of 20000 Da (Fig. 2; Table 2). Phosphorylation of most of the proteins on day 0 was significantly lower with dbcAMP than observed with LH/MIX, especially for the 17000 Da protein. However, dephosphorylation of the 20000 Da protein was the same with LH/MIX or dbcAMP (Table 2). For comparison of the effects of LH/MIX and dbcAMP on pregnenolone production and (de)phosphorylation of proteins see Fig. 3. LH/MIX-stimulated phosphorylation of the 17000 Da protein decreased more rapidly than (de)phosphorylation of the five other LH-dependent phosphoproteins (see: Table 2). Changes in incubation temperature (37° versus 32°C) had no significant effects on protein phosphorylation.

cAMP-dependent protein kinase

The decreased phosphorylation of the LH-dependent phosphoprotein of 57000 Da (the regulatory subunit of the type II cAMP-dependent protein kinase; cf. Cooke et al., 1979) suggested a decrease in activity of cAMP-dependent protein kinase. Hence, protein kinase enzyme activity was determined with histones as substrate.

Table 1 Stimulated pregnenolone production during short-term culture of tumour Leydig cells

		+ LH/MIX (µg/60 min/mg protein)	+ dbcAMP (µg/60 min/mg protein)	+ 25-hydroxycholesterol (µg/60 min/mg protein)
day 0	32°C	0.23 ± 0.11 (100)	0.47 ± 0.13 (100)	1.68 ± 0.26 (100)
	37°C	0.31 ± 0.13 (100)	0.67 ± 0.06 (100)	2.00 ± 0.23 (100)
day 1	32°C	0.16 ± 0.05 (72)	0.35 ± 0.09 (74)	1.57 ± 0.25 (93)
	37°C	0.11 ± 0.10 (36)	0.31 ± 0.13 (46)	1.45 ± 0.27 (72)
day 2	32°C	0.03 ± 0.02 (13)	0.09 ± 0.07 (20)	0.74 ± 0.48 (44)

Tumour Leydig cells were incubated in duplicate for 60 min in the presence of LH/MIX, dbcAMP or 25-hydroxycholesterol, and inhibitors of pregnenolone metabolism. Control productions were subtracted. Results are means ± S.D. ($n = 4$). After incubation of cells at 37°C instead of 32°C stimulated pregnenolone production was significantly increased on day 0 ($p < 0.005$) and significantly decreased on day 1 ($p < 0.01$; $n = 12$). DbcAMP-stimulated pregnenolone production was significantly higher than the LH/MIX-stimulated pregnenolone production: $p < 0.05$. The 25-hydroxycholesterol-stimulated pregnenolone production (in per cent) was significantly higher than LH/MIX- and dbcAMP-stimulated pregnenolone production on day 1 and day 2: $p < 0.05$. The values in parentheses give the effect of the adjuvant expressed as the percentage of the effect on day 0.

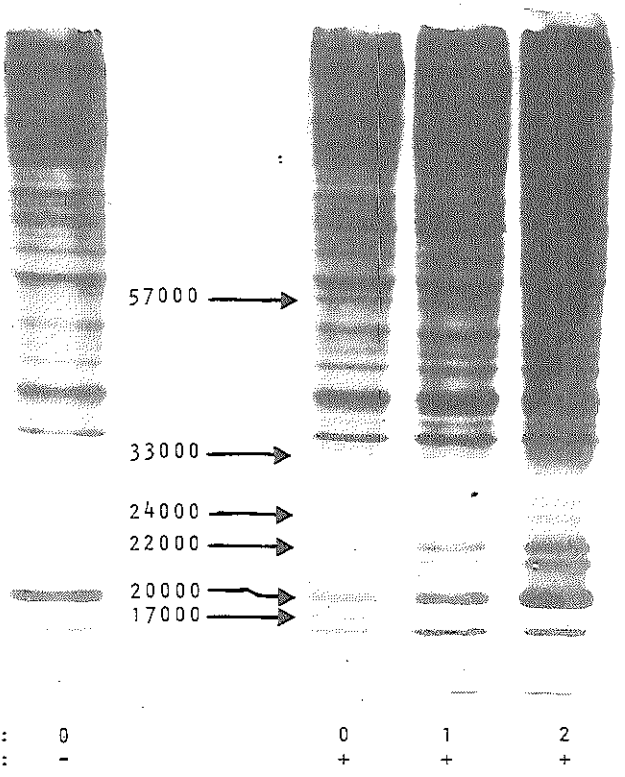

Fig. 2. Autoradiogram showing the effects of culture on LH-dependent protein phosphorylation. Tumour Leydig cells were cultured for 2 days and incubated for 60 min with (^{32}P)P_i on each day of culture in the absence (-) or presence (+) of LH/MIX (see: Materials and Methods). Phosphorylated proteins were isolated and separated by SDS-polyacrylamide gel electrophoresis. For the control samples only the autoradiogram of phosphorylated proteins isolated on day 0 is shown. Numerical values denote molecular masses in Daltons.

Table 2 LH/MIX- and dbcAMP-stimulated phosphorylation and dephosphorylation (%) of proteins during short-term culture of tumour Leydig cells

Molecular mass		+ LH/MIX (% peak height)	+ dbcAMP (% peak height)
17000 Da	day 0	19.1 ± 5.2[a,b] (100)	4.1 ± 1.1[a] (100)
	day 1	10.5 ± 0.4[b] (58)	1.8 ± 1.6 (44)
	day 2	1.7 ± 1.6 (9)	1.8 ± 2.5 (44)
22000 Da	day 0	11.2 ± 3.4[a,a] (100)	8.2 ± 5.0 (100)
	day 1	5.6 ± 1.7[a] (50)	8.1 ± 5.8 (99)
	day 2	5.1 ± 5.7 (46)	4.8 ± 4.0 (59)
24000 Da	day 0	9.4 ± 1.7[b] (100)	4.6 ± 3.2 (100)
	day 1	4.5 ± 2.7 (48)	2.7 ± 2.3 (59)
	day 2	3.5 ± 5.3 (37)	2.7 ± 1.4 (59)
33000 Da	day 0	13.4 ± 3.5[a,a] (100)	8.1 ± 1.2[b,b] (100)
	day 1	8.9 ± 4.0[b] (66)	6.7 ± 1.5[a] (83)
	day 2	3.1 ± 0.4 (23)	2.6 ± 1.2 (32)
57000 Da	day 0	7.6 ± 3.3[a,a] (100)	6.1 ± 2.0[a,a] (100)
	day 1	5.8 ± 2.4[a] (76)	2.9 ± 1.2[b] (47)
	day 2	4.3 ± 2.1 (57)	3.2 ± 0.3 (52)
20000 Da*	day 0	25.9 ±11.0[a] (100)	27.3 ± 9.2[a] (100)
	day 1	18.9 ± 8.2[a] (73)	19.2 ± 5.0[a] (70)
	day 2	10.8 ± 2.4[b] (42)	16.1 ± 1.0[b] (59)

Tumour Leydig cells were incubated at 32°C with (^{32}P)P_i in the presence or absence of LH/MIX or dbcAMP. Densitogram tracings of autoradiograms were obtained after electrophoresis of phosphorylated proteins. Phosphorylation of LH-dependent phosphoproteins was expressed relative to the phosphorylation of an LH-independent phosphoprotein of 40000 Da as a peak height ratio (Bakker et al., 1981). Values shown are stimulated (de)phosphorylation of proteins (control values subtracted). Peak height ratios of protein phosphorylation not stimulated by LH/MIX or dbcAMP on day 1 and day 2 were less than 10% different from the peak height ratio on day 0. Results are means ± S.D. of three different cell preparations. Statistical analysis was performed with the corresponding control incubation on each day of culture. a, $p < 0.05$; b, $p < 0.01$. DbcAMP-stimulated phosphorylation of proteins was significantly lower on day 0 relative to LH/MIX-stimulated phosphorylation (17000 Da, $p < 0.025$; four other phosphoproteins: $p < 0.05$). Numbers in parentheses denote the effect of the adjuvant expressed as the percentage of the effect on day 0.

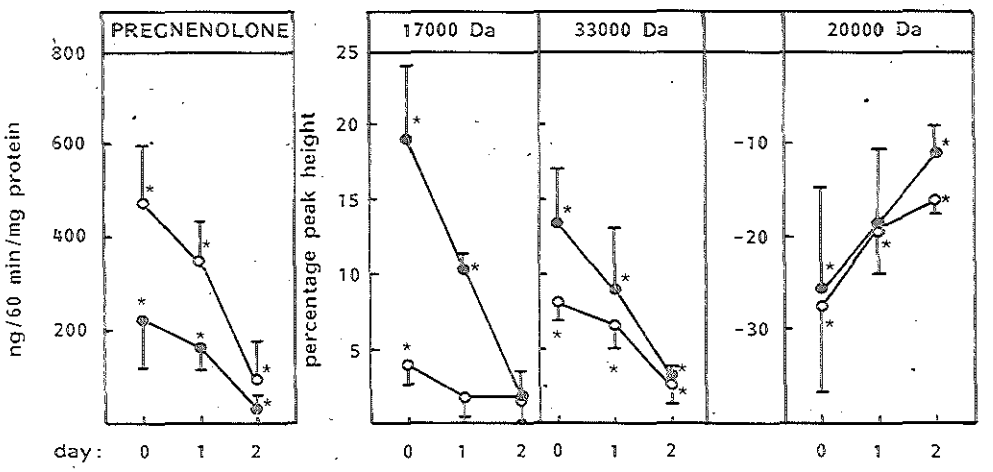

Fig. 3. Effect of culture period on LH/MIX (●)- and dbcAMP (o)-stimulated pregnenolone production and phosphorylation/dephosphorylation of proteins in tumour Leydig cells at 32°C. Results are means ± S.D. (n = 3-4; from Tables 1 and 3).
*, significantly different ($p < 0.05$) from control incubations on each day.

cAMP-independent and cAMP-stimulated activity of protein kinase declined continuously during the two days culture period (Table 3). Protein kinase enzyme activity (always assayed at 37°C) was not significantly decreased when cells were cultured for 24 h at 37°C instead of 32°C (Table 3). The decline in stimulated phosphorylation of the 57000 Da protein in the presence of LH/MIX occurred in parallel with the decline in activity of cAMP-independent and cAMP-dependent protein kinase enzyme activity (Fig. 4).

Table 3. Activity of cAMP-dependent protein kinase during short-term culture of tumour Leydig cells at 32°C and 37°C

	control		with added cAMP	
	32°C	37°C	32°C	37°C
day 0	9.2 ± 2.9 (100)	9.9 ± 4.5 (108)	45.6 ± 26.2 (100)	48.9 ± 37.7 (107)
day 1	6.5 ± 2.4 (71*)	6.0 ± 1.5 (65*)	35.0 ± 22.8 (77*)	29.8 ± 18.8 (65*)
day 2	5.5 ± 1.9 (60*)	n.d.	25.4 ± 12.3 (56*)	n.d.

Values are incorporation of 10^{-3} x ^{32}P-cpm/10 min/mg protein. Stimulation of kinase activity by cAMP was 4-6-fold. Results are means ± S.D. obtained with three different cell preparations and for each cell preparation three different enzyme assays. Numbers in parentheses denote the activity expressed as the percentage of the activity on day 0 at 32°C.

*, significantly different from activity on day 0, $p < 0.05$ (paired t-test);

n.d. = not determined.

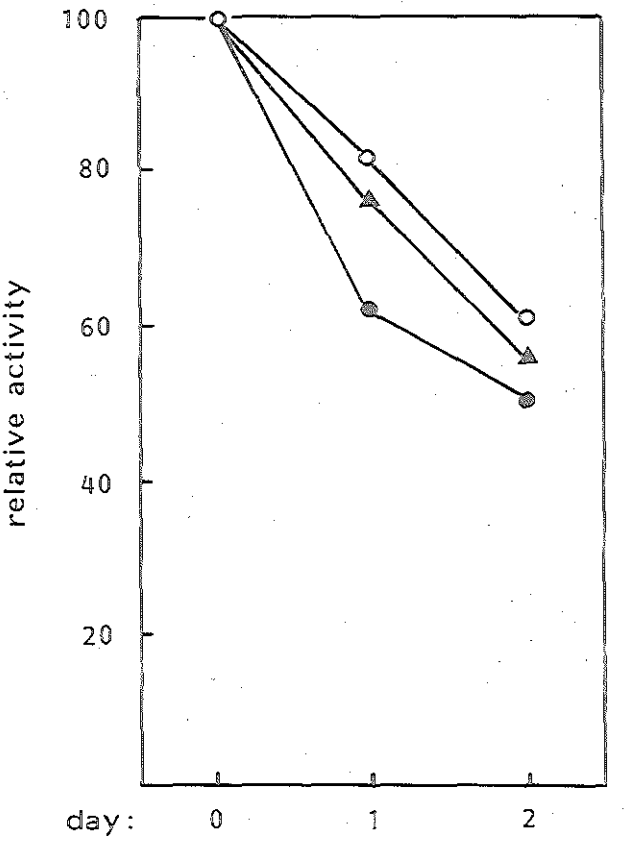

Fig. 4. Effect of culture on the activity on cAMP-dependent protein kinase (control, ●; with cAMP, o) and LH/MIX-stimulated phosphorylation of the 57000 Da protein (▲). All values shown for day 1 and day 2 differ significantly from values at day 0 ($p < 0.05$). Results are means values ($n = 3$).

DISCUSSION

Specific phosphoproteins are probably important for regulation of hormone-dependent steroid production. In tumour Leydig cells, LH stimulation resulted in increased pregnenolone production and increased phosphorylation of five proteins of 17000, 22000, 24000, 33000 and 57000 Da (Bakker et al., 1981). To investigate which LH-dependent phosphoproteins might be essential for regulation of steroid production, changes in hormone-dependent pregnenolone production and protein phosphorylation were studied during short-term culture of tumour Leydig cells.

During short-term culture of tumour Leydig cells LH-dependent pregnenolone production and (de)phosphorylation of specific proteins declined in parallel. Incubation of cells at 37°C instead of 32°C resulted in a significant increase in pregnenolone production on day 0, whereas after 24 h incubation at 37°C pregnenolone production was significantly lower. Cell viability was apparently not changed, because there were no morphological indications for cell degeneration when cells had been cultured for more than seven days. Moreover, protein content and incorporation of ^{14}C-labelled amino acids into proteins increased during the culture period, indicating that cells also maintained general functional activities. The constant pattern of hormone-independent protein phosphorylation suggests that also many specific functional activities are maintained during the culture period.

From these observations we have concluded, that the changes which occurred in the LH-dependent protein phosphorylation and steroid production are rather specific and cannot be attributed to non-specific effects resulting from cell damage.

Addition of dbcAMP instead of LH/MIX to tumour Leydig cells on day 0 resulted in significantly higher stimulated pregnenolone production (see also: Carr et al. 1981) and significantly lower phosphorylation of hormone-dependent phosphoproteins. The greater stimulation of protein phosphorylation by LH/MIX (especially the 17000 Da protein), when compared to dbcAMP, may reflect stimulation of other than cAMP-dependent protein kinase systems (Nishizuka et al., 1979). However, it is difficult to explain the relatively high protein phosphorylation and low steroid production in the presence of LH/MIX in comparison with the relatively low protein phosphorylation and high steroid production induced by dbcAMP. It may be possible that high doses of LH cause, in addition to stimulatory actions, also inhibitory actions.

A remarkable feature of the hormonal response of cultured tumour Leydig cells was the dephosphorylation of a 20000 Da protein in the

presence of LH/MIX or dbcAMP. A similar observation has been described for rat adrenal cells incubated with ACTH (Koroscil & Gallant, 1980). The LH/MIX-stimulated dephosphorylation of the 20000 Da protein also decreased during culture. Similar to observations with adrenal cells (Koroscil & Gallant, 1980) the 20000 Da protein in tumour Leydig cells is also present in the cytosol fraction (results not shown).

LH-dependent phosphorylation of the 17000 Da nuclear protein (cf. Bakker et al., 1981) decreased faster during culture than (de)phosphorylation of all other LH-dependent phosphoproteins. In combination with the relatively slow phosphorylation of the 17000 Da protein after addition of LH/MIX (Bakker et al., 1981), these observations could indicate that phosphorylation of the 17000 Da protein is not directly involved in the acute regulation of steroid production by LH.

The capacity of the CSCC activity in tumour Leydig cells estimated with 25-hydroxycholesterol (cf. Mason & Robidoux, 1978; Alsema et al., 1980; Toaff et al., 1982) was significantly higher and decreased significantly less than the DH/MIX- or dbcAMP-stimulated pregnenolone production. Thus, the CSCC capacity is probably not rate-limiting for the LH/MIX- (or dbcAMP-) stimulated steroid production, but apparently the control of the CSCC changes during culture of the Leydig cells. Both the LH/MIX- and dbcAMP-stimulated steroid production decreased in culture and this suggests that the major changes relevant to the control of CSCC occur after formation of cAMP.

Specific protein phosphorylation declined in culture, and the similarities in the decline of hormone-stimulated phosphorylation and dephosphorylation of specific proteins indicate that a defect at the level of protein phosphorylation developed during culture. The decline in phosphorylation of the LH-dependent phosphoprotein of 57000 Da, which has been identified as the regulatory subunit of the type II cAMP-dependent protein kinase (cf. Cooke et al., 1979), suggested that the activity of cAMP-dependent protein kinase had decreased. Estimation of protein kinase enzyme activity in broken cells showed a significant decrease in activity of cAMP-dependent and -independent protein kinase. The decline in protein kinase activities parallelled the decline in LH-dependent phosphorylation of the 57000 Da protein, suggesting that the decline in protein kinase activity might be caused by decreased amounts of the cAMP-dependent protein kinase.

Stimulation of CSCC in Leydig cells by LH is the final step in a cascade of events and amplification steps. This cascade includes cAMP formation, activation of protein kinase, phosphorylation of proteins and

protein synthesis. Various dose-response relationships of these activities have been compared. It has been shown that the dose-response curve for hormone-dependent cAMP production and steroid production are at completely different hormone levels (Cooke et al., 1976; Schumacher et al., 1979; Darbon et al., 1981; review by Schimmer, 1980). The cAMP concentrations for binding to or activation of protein kinase and steroid production are more comparable (Cooke et al., 1976; Podesta, 1978; Schumacher et al., 1979), but still far from perfect. However, dose-response curves for protein phosphorylation and steroid production coincide almost perfectly (Cooke et al., 1977; Koroscil & Gallant, 1980; Darbon et al., 1981; Gonzalez-Martinez et al., 1982). The kinetics of protein phosphorylation and steroid production also correlate (Koroscil & Gallant, 1980; Bakker et al., 1981; Darbon et al., 1981). Measurement of endogenous protein phosphorylation therefore appears to represent quantitatively the most reliable parameter for initial events occurring after hormonal stimulation of Leydig cells and it shows the best correlation with the functional response, i.e. with steroid production.

The extent of phosphorylation of most of the LH-dependent proteins after two days culture was decreased to approx. 40%, whereas steroid production was decreased to 13%. This difference may indicate that other factors in between protein phosphorylation and activation of CSCC, such as synthesis of specific proteins, may also decrease concomitant with the accompanying effects on the CSCC activity.

Cells incubated for 1 h at 37°C (day 0) were more active in pregnenolone production than cells at 32°C, as can be expected from general temperature effects on enzyme activities. In contrast, cells incubated for 24 h at 37°C produced less amounts of steroids than cells incubated for 24 h at 32°C. These results may indicate that the specific processes of protein kinase and protein phosphorylation are also stimulated at higher temperature. These small temperature effects, however, could not be detected in protein phosphorylation and protein kinase activity and are probably masked by the experimental conditions.

Phosphorylation of Leydig cell proteins has been documented for freshly isolated rat Leydig cells (Cooke et al., 1977; Bakker et al., 1981), in rat Leydig cell extracts (Cooke et al., 1979; Dufau et al., 1981) and in primary cultures of porcine Leydig cells (Gonzalez-Martinez et al., 1982).

In cultured mouse Leydig cells dbcAMP was unable to maintain testosterone responsiveness for more than three days, which suggested a block in the steroidogenic pathway (Hunter et al., 1982). However, decreased activity of cAMP-dependent protein kinase might partly explain

the results with mouse Leydig cells. The present results do not indicate which factor(s) are necessary to maintain the activity of cAMP-dependent protein kinase in cultured cells. Moreover, tumour Leydig cells isolated from mature male rats seven days after hypophysectomy, responded to LH/MIX or dbcAMP (results not shown) comparable to cells isolated from sham-operated animals. These results indicate that LH is not important for maintenance of protein kinase but only for activation (see also: Hsueh, 1980).

In conclusion, decreased activities of at least one important phosphoprotein, i.e. the (regulatory subunit of) cAMP-dependent protein kinase, significantly contribute to the decreased steroid production of cultured (tumour) Leydig cells. It is not clear, however, which factor(s) are important for the maintenance of this enzyme.

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Appendix Paper IV

(Submitted to Molec. Cell. Endocr.)

POSSIBLE FUNCTIONS OF LH-DEPENDENT PHOSPHOPROTEINS AND PROTEIN SYNTHESIS IN THE REGULATION OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN RAT TUMOUR LEYDIG CELLS

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SYNOPSIS

Stimulation of tumour Leydig cells with lutropin/1-methyl-3-isobutylxanthine (LH/MIX; 1000 ng/ml and 0.25 mM, resp.) resulted in increased phosphorylation of seven proteins of 17000, 22000, 24000, 33000, 43000, 57000 and 76000 Da, and in dephosphorylation of a protein of 20000 Da. None of these phosphoproteins was present in mitochondria. The possible role of these extra-mitochondrial LH-dependent phosphoproteins for steroid production has been investigated in more detail.

Addition of actinomycin D (10 µg/ml) to tumour Leydig cells did not affect LH-dependent pregnenolone production within 1 h. Hence, synthesis of (m)RNA and LH-dependent phosphorylation of the nuclear phosphoprotein of 17000 Da appear not important for acute regulation of steroid production. The 20000, 22000, 43000, 70% of the 57000, and the 76000 Da phosphoproteins were present in the cytosol fraction. The phosphorylation of the 76000 Da concomitant with dephosphorylation of the 20000 Da protein suggested that these (phospho)proteins represent the microfilament proteins myosin light-chain (the 20000 Da protein) and its kinase (the 76000 Da protein). Cytochalasin B (50 µM) inhibited control and LH-dependent pregnenolone production by approx. 50%, but had no effect on protein phosphorylation.

Addition of LH (100 ng/ml) to perifused Leydig cells from immature rats preincubated with cycloheximide (89 µM) did not cause a transient stimulation of pregnenolone production, suggesting that relatively stable precursor protein(s) are probably not obligatory for regulation of steroid production.

The 24000, 33000 and 30% of the 57000 Da phosphoproteins were present in the microsomal fraction of tumour leydig cells. Effects of LH on synthesis of specific proteins could not be detected using SDS-polyacrylamide gel electrophoresis of radioactively labelled proteins. However, increasing concentrations of cycloheximide (0.2 - 0.8 µM) inhibited LH-dependent pregnenolone production significantly more than unstimulated steroidogenesis. These specific effects of low concentrations of cycloheximide occur when hormones regulate initiation of protein synthesis.

In conclusion, the present results suggest that the LH-dependent phosphoproteins may play a role in the regulation of the cytoskeleton and specific synthesis of presently unidentified rapidly-turning-over protein(s) which ultimately influence(s) the mitochondrial cholesterol-side-chain cleavage (CSCC) activity.

Abbreviations used: ACTH, adrenocorticotropic hormone;
cAMP, adenosine cyclic 3',5'-monophosphate;
CSCC, cholesterol side-chain cleavage;
25-hydroxycholesterol, 5-cholestene-3β,25-diol;
SDS, sodium dodecylsulphate.

INTRODUCTION

Addition of lutropin/1-methyl-3-isobutylxanthine (LH/MIX; 1000 ng/ml and 0.25 mM, resp.) to tumour Leydig cells resulted in increased phosphorylation of five proteins of 17000, 22000, 24000, 33000 and 57000 Da (Bakker et al., 1981). A 20000 Da protein was dephosphorylated by LH/MIX (Bakker et al., 1983). The similarities in the kinetics of phosphorylation, dephosphorylation and pregnenolone production after addition of LH/MIX under different experimental conditions (Bakker et al., 1981, 1983) suggested that all LH-dependent phosphoproteins may be essential for LH-regulation of steroid production in tumour Leydig cells. None of these specific LH-dependent phosphoproteins was present in the mitochondria, which indicates that regulation of cholesterol side-chain cleavage (CSCC) activity by these specific phosphoproteins occurs indirectly.

Hormonal regulation of steroidogenesis requires the continuous synthesis of protein(s) (Garren et al., 1965; Schulster et al., 1974; Cooke et al., 1975; Farese et al., 1980; Bakker et al., 1982; Mori & Marsh, 1982). The amount of the specific regulator proteins may be controlled by changes in the rate of synthesis of mRNA, transformation of relatively stable, pre-existing precursor proteins or via changes in the rate of initiation or elongation of mRNA translation. LH action on tumour Leydig cells involves phosphorylation of the nuclear 17000 Da protein (Bakker et al., 1981) as well as phosphorylation of the ribosomal protein S6 (Bakker et al., 1982). Hence, LH-dependent phosphoproteins may be involved in synthesis of the protein factor(s) required for LH-dependent steroid production at the level of DNA transcription and/or mRNA translation. Moreover, phosphoproteins may influence the cytoskeleton (e.g. Janis et al., 1980; Bhalla et al., 1982), which has been shown to be important for steroid production (Crivello & Jefcoate, 1978; Hall et al., 1979; Silavin et al., 1980).

The present study was undertaken to investigate which subcellular activities may be regulated by the indicated LH-dependent phosphoproteins and whether these activities might be involved in the regulation of steroid production.

MATERIALS AND METHODS

Chemicals used, procedures for isolation of Leydig cells from immature rat testes and 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 previously (Bakker et al., 1981, 1982). (4,5-^{3}H)Leucine (58 Ci/mmol) and (5-^{3}H)uridine (5 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Actinomycin D was obtained from Boehringer, Mannheim, Germany. Cytochalasin B was from Sigma, St. Louis, MI, U.S.A. 5-Cholestene-3β,25-diol (25-hydroxycholesterol) was from Steraloids Inc., Wilton, NH, U.S.A. Experiments were performed generally with 1-2 x 10^{6} cells preincubated at 32°C for 1 h, and incubated for another hour.

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.

Perifusion of immature Leydig cells

Perifusion of immature Leydig cells was performed as described previously (Rommerts et al., 1982). Immature Leydig cells were preincubated on a special support in a Petri dish prior to perifusion. Perifusion of cells was performed with Minimum Essential Medium containing 1% foetal calf serum and either cycloheximide (89 µM) or cycloheximide plus LH (100 ng/ml) as explained in the legend to Figure 2.

Effects of low concentrations of cycloheximide

The effects of low concentrations of cycloheximide (0.2-0.8 µM) on protein synthesis and LH-dependent pregnenolone production were studied with tumour Leydig cells. Tumour Leydig cells were incubated with ^{14}C-amino acids for 30 min or with inhibitors of pregnenolone metabolism for 60 min (Bakker et al., 1982).

Subcellular fractionation

Microsomes (199000 x g_{av} pellet) and cytosol (199000 x g_{av} supernatant) were isolated from the post-mitochondrial supernatant prepared according to Bakker et al. (1981). The medium for homogenization of tumour Leydig cells (Freienstein & Blobel, 1974) 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 rpm - 199000

g_{av}) for 3 h 40 min, or a Beckman Airfuge (100000 rpm - 132000 g_{av}) for 17 min was used.

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 (Bakker et al., 1981).

RESULTS

Subcellular fractionation

Subfractionation of the post-mitochondrial supernatant isolated from tumour Leydig cells showed that the LH-dependent phosphoproteins of 24000 and 33000 Da appeared to be concentrated in the microsomal fraction, whereas the 20000, 22000, 43000 and 76000 Da phosphoproteins were concentrated in the cytosol fraction (Fig. 1 and Table 1). The 57000 Da was

Table 1 Subcellular localization of lutropin-dependent phosphoproteins in microsomal and cytosol fractions of tumour Leydig cells

Molecular mass in Daltons	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 ± S.D. (from four different subcellular fractionations).

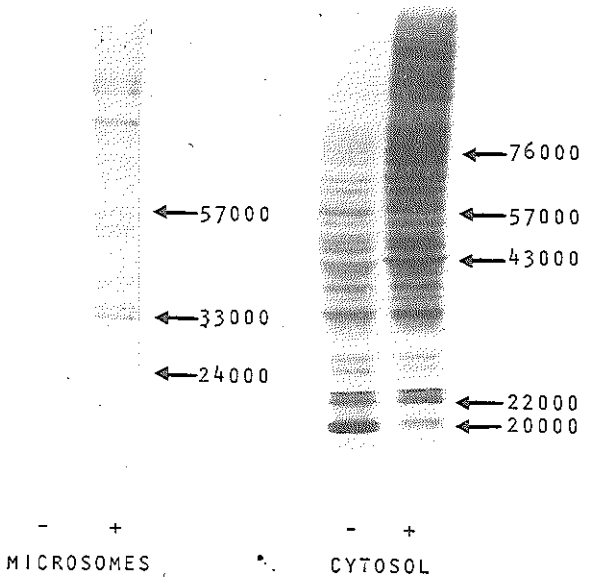

Fig. 1. Autoradiogram showing the localization of LH-dependent phosphoproteins in microsomal and cytosol fractions of tumour Leydig cells.
Tumour Leydig cells (15×10^6) were incubated in the presence of $^{32}P\text{-}P_i$ without (-) or with (+) LH/MIX. Microsomes and cytosol were obtained using the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

for 30% present in microsomes and for 70% in the cytosol (Table 1). Almost no mutual contamination of the fractions was observed and the pattern of LH-independent phosphoproteins was completely different. Phosphorylation of the 20000 Da was peculiar, because under the influence of LH/MIX phosphorylation of this protein decreased.

The 43000 and 76000 Da proteins were clearly present in the cytosol fractions, but were difficult to observe in preparations of phosphorylated proteins from intact cells. In addition, the intensity of especially the 43000 Da phosphoprotein band in the autoradiogram was dependent on the fractionation procedure, as the band was more pronounced after isolation, using the Airfuge (centrifugation time 17 min) than using the normal ultracentrifuge (centrifugation time 3 h 40 min).

Experiments with cytochalasin B

To study a possible involvement of microfilaments in LH-dependent steroid production, the effect of cytochalasin B on LH-dependent

pregnenolone production and phosphorylation of proteins was estimated. Cytochalasin B inhibited control and LH/MIX-stimulated pregnenolone production by approx. 60% and 45% resp. (Table 2), whereas cytochalasin B inhibited pregnenolone production in the presence of 25-hydroxycholesterol for not more than 15%. No effect could be observed on (de)phosphorylation of proteins under control and LH/MIX-stimulated conditions (results not shown).

Experiments with actinomycin D

The possible involvement of (m)RNA synthesis in LH action was studied with actinomycin D. 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 production (Table 3).

Perifusion of immature Leydig cells

The presence of a relatively stable precursor protein which could give rise to formation of protein(s) with a short half-life after administration of LH was estimated during perifusion of immature Leydig cells in the presence of cycloheximide. If a stable precursor protein plays a role, LH stimulation of preincubated Leydig cells in the presence of cycloheximide would induce a transient increase in steroid production. However, no such transient increase in pregnenolone production could be demonstrated apart from a slightly increased pregnenolone production during the perifusion, which was LH-independent (Fig. 2).

Protein synthesis and pregnenolone production

Protein synthesis in tumour Leydig cells was also investigated by comparison of SDS patterns of newly synthesized ^{14}C-labelled proteins from cells incubated for 15 min with or without LH/MIX. However, no effects of LH/MIX on the qualitative pattern of protein synthesis could be observed (results not shown).

Quantitative effects of various low concentrations of cycloheximide on incorporation of ^{14}C-labelled amino acids into proteins and pregnenolone production in tumour Leydig cells were also determined. Overall protein synthesis was equally inhibited in control and LH/MIX-stimulated cells, whilst inhibition of LH-dependent pregnenolone production was significantly higher than inhibition of control pregnenolone production ($p < 0.005$; Fig. 3). The lines drawn in Fig. 3A do not run in parallel ($p < 0.01$), indicating that increasing doses of cycloheximide resulted in a greater inhibitory effect on stimulated cells than on unstimulated cells.

Table 2 Effect of cytochalasin B (50 µM) on pregnenolone production in tumour Leydig cells

	no cytochalasin B (ng/h/mg protein)	with cytochalasin B (ng/h/mg protein)	% 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 percentage 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 ± S.D. obtained in three different experiments with duplicate incubations.

Table 3 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, followed by incubation for 1 h at 37°C in the presence of ^{3}H-uridine, ^{3}H-leucine or inhibitors of pregnenolone metabolism, and with or without actinomycin D (10 µg/ml). The average percentage decrease was calculated from individual values obtained within each experiment. Results are means ± S.D. with the number of different cell preparations in parentheses. Basal and LH-dependent pregnenolone production (in ng/h per mg protein) were 185 ± 89 and 622 ± 400 (mean ± S.D.; n = 6).

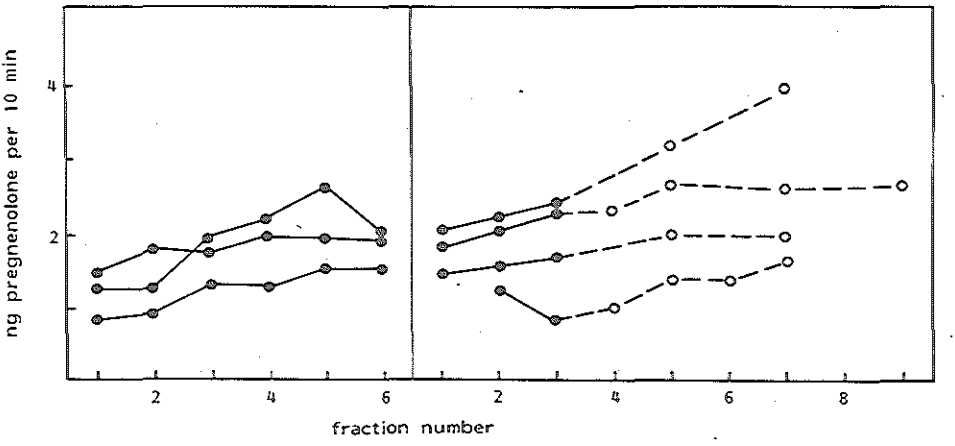

Fig. 2. Effect of cycloheximide (89 µM) on LH-dependent pregnenolone production during perifusion of Leydig cells isolated from immature rat testes
Perifusion of 1 h preincubated cells was performed with medium containing cycloheximide (solid lines) or with medium containing cycloheximide plus LH (100 ng/ml) (dashed lines). Results shown are the amounts of pregnenolone released into the perifusion medium during 10 min periods. Results obtained with the same group of cells are connected via the (solid or dashed) lines. Results shown were obtained with cells from three different cell preparations.

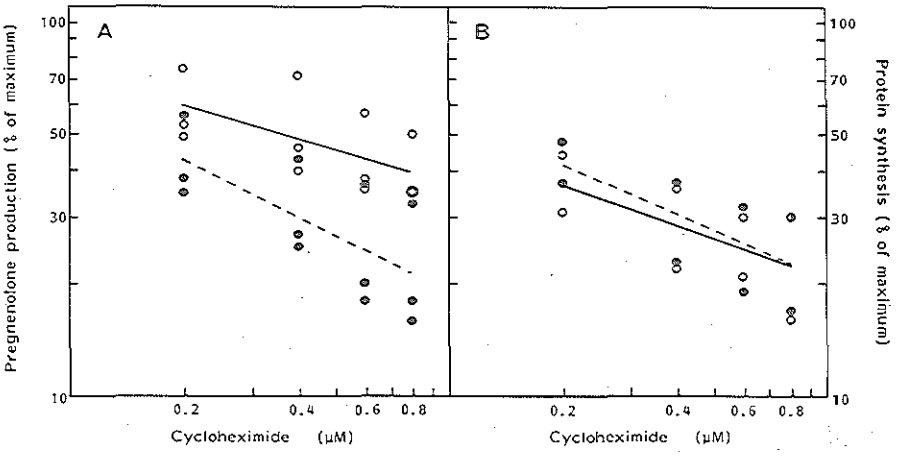

Fig. 3. Effects of low concentrations of cycloheximide on pregnenolone production (A) and incorporation of ^{14}C-labelled amino acids into proteins (B) in tumour Leydig cells
Tumour Leydig cells were preincubated for 1 h and incubated with various doses of cycloheximide, and with (dashed lines) or without (solid lines) LH/MIX. Incubations were carried out for 60 min for estimation of pregnenolone (A) or for 30 min with ^{14}C-labelled amino acids (B). Results are expressed as the percentage of the activity in the absence of cycloheximide. Mean values of duplicate incubations from three (A) or two (B) different cell preparations are shown. Inhibition of LH-dependent pregnenolone production was significantly higher than inhibition of control pregnenolone production ($p < 0.005$). Lines drawn in panel A do not run in parallel ($p < 0.01$). Statistical analysis was performed with Student's paired t-test.

DISCUSSION

Protein synthesis and cellular microfilaments appear to be required for LH-dependent steroid production, and the extra-mitochondrial LH-dependent phosphoproteins of 17000, 22000, 24000, 33000 and 57000 Da in tumour Leydig cells (Bakker et al., 1981) may play a role in regulation of protein synthesis and/or the cytoskeleton.

We have shown previously, that the LH-dependent phosphoprotein of 17000 Da is localized in the nucleus (Bakker et al., 1981) and this may suggest that nuclear activities are required for the effect of LH on protein synthesis and LH-stimulated steroid production. Inhibition of (m)RNA synthesis with actinomycin D, however, had no effect on LH-dependent pregnenolone production. This may indicate that (m)RNA synthesis is not required for the acute steroidogenic response to LH in tumour Leydig cells, similar to observations made with adrenal tissue for ACTH (Garren et al., 1965), testis Leydig cells for LH (Cooke et al., 1979b) and ovarium follicles for LH (Losier & YoungLai, 1981).

Fractionation of the post-mitochondrial supernatant isolated from tumour Leydig cells showed the presence of LH-dependent phosphoproteins in cytosol and microsomes. LH-dependent phosphoproteins of 20000, 22000, 43000, 57000 and 76000 Da were present in the cytosol. The combination of the molecular masses, the cytosol localization, the phosphorylation of the 76000 Da protein coinciding with dephosphorylation of the 20000 Da protein, suggests that the 20000 and 76000 Da phosphoproteins could be similar to myosin light-chain and myosin light-chain kinase respectively (Adelstein et al., 1978; Bhalla et al., 1982). The LH-dependent phosphoprotein of 43000 Da present in the cytosol and actin share the same molecular mass (e.g. Riddle et al., 1979a). Leydig cells isolated from immature and mature rat testes also showed LH-dependent phosphorylation of a 76000 Da protein (see: Bakker et al., 1982) and dephosphorylation of a 20000 Da protein (unpublished results). These results suggest that the 20000, 43000 and 76000 Da phosphoproteins may be related to microfilaments. The LH-dependent phosphorylation of these putative microfilaments proteins might indicate that activation of microfilaments via phosphorylation of proteins could be essential for hormone-dependent steroid production (Crivello & Jefcoate, 1978; Hall et al., 1979; Silavin et al., 1980).

This hypothesis was furher tested by incubation of tumour Leydig cells with cytochalasin B. Cytochalasin B significantly inhibited both control and LH-dependent pregnenolone production, whereas a rather small, although significant, effect on 25-hydroxycholesterol-stimulated pregnenolone production and no effect on phosphorylation of proteins could

be demonstrated. Pregnenolone production in the presence of 25-hydroxycholesterol gives an estimation of the CSCC activity (cf. e.g. Toaff et al., 1982). The (apparent) unaffectedness of CSCC and protein phosphorylation in the presence of cytochalasin B suggests that CSCC and activation of cAMP-dependent protein kinase do not involve microfilaments, which are influenced by cytochalasin B.
The effect of cytochalasin B on unstimulated pregnenolone production may be explained by the fact that pregnenolone production in tumour Leydig cells is slightly stimulated by other factors than LH. This has been discussed earlier to explain the inhibitory effect of cycloheximide on control pregnenolone production (Bakker et al., 1982).

Protein synthesis is necessary for hormone-dependent steroid production (e.g. Schulster et al., 1974; Cooke et al, 1975). Since nuclear processes are probably not involved in the acute steroidogenic response (see above), the protein factor(s) required either could be present as a precursor protein(s) as proposed by Cooke et al. (1979a), or should be newly synthesized as proposed by Garren et al. (1965).

Cooke et al. (1979a) proposed that stimulation of steroid production in testis Leydig cells may involve transformation of a stable precursor protein(s) into an active protein with a short half-life. It was suggested that this precursor protein(s) could be synthesized independent of LH action. If this is true, the presence of precursor protein(s) might enable a transient steroid response when protein synthesis is inhibited by e.g. cycloheximide. However, the perifusion experiments with immature Leydig cells in the present study did not show experimental eidence for the presence of a regulator protein(s). Hence, if such precursor protein(s) do exist, these are apparently of minor importance for LH-dependent steroid production.

Hormone-dependent steroid production may thus rely on newly synthesized rapidly-turning-over protein(s) (cf. Garren et al., 1965). We have shown, in agreement with others, that overall protein synthesis in steroidogenic tissues and cells is not increased shortly after addition of hormone (Garren et al., 1965; Cooke et al., 1975; Bakker et al., 1982; YoungLai & Osoko, 1982). Moreover, we and others have not been able to demonstrate acutely increased synthesis of specific proteins after analysis of labelled proteins with SDS-polyacrylamide gel electrophoresis (Janszen et al., 1977; Losier & YoungLai, 1981; YoungLai & Osoko, 1982). An effect of LH on induction of a specific protein, however, could be shown after 2 h (Janszen et al., 1977) (see also: Nakamura et al., 1978).

Stimulation of (tumour) Leydig cells with LH results in phosphorylation of ribosomal protein S6 (Bakker et al., 1982) and the

microsomal 24000 Da protein. The combined action of these phosphoproteins could be responsible for a specific effect on translation. Regulation of protein synthesis occurs at the level of initiation of translation (Lodish, 1976; Hunt, 1980a), as described for effects of insulin on overall protein synthesis (Monier & Le Marchand-Brustel, 1982) and for the effect of dibutyryl cAMP on synthesis of tyrosine aminotransferase (Snoek et al., 1981). Phosphorylation of initiation factor eIF-2 in reticulocytes appears to be clearly involved in the regulation of haemoglobin synthesis (see reviews by Austin & Clemens, 1980; Hunt, 1980b). In this context the 24000 Da LH-dependent phosphoprotein present in microsomes may have properties similar to initiation factor eIF-4E (the mRNA-cap binding protein; see: Thomas et al., 1981). However, no further direct experimental evidence for an effect on protein synthesis could be obtained.

Low concentrations of cycloheximide have been used to study the control mechanisms for synthesis of a labile protein required for growth of cultured fibroblasts (Brooks et al., 1977; Riddle et al., 1979b) and induction of tyrosine aminotransferase in rat hepatoma cells (Snoek et al., 1981). Cycloheximide inhibits elongation of translation (Siegel & Sisler, 1965). Inhibition of protein synthesis in the presence of cycloheximide occurs when the rate of elongation becomes less than the rate of initiation, and an apparently greater inhibition of protein synthesis will be observed when at the same low concentration of cycloheximide the rate of initiation of translation is increased, e.g. by hormone treatment (Snoek et al., 1981; Monier & Le Marchand-Brustel, 1982). Incubation of tumour Leydig cells with low concentrations of cycloheximide resulted in a significantly higher inhibition of LH/MIX-stimulated pregnenolone production as compared to control pregnenolone production. However, there was no difference in inhibition of overall protein synthesis in control and LH/MIX-stimulated cells. In analogy with the results described by Snoek et al. (1981), the significantly higher inhibition of cycloheximide may indicate, although indirectly, increased initiation of translation of a specific mRNA(s) coding for the rapidly-turning-over protein (s). The LH-dependent phosphoproteins of 24000 (eIF-4E?) and 33000 Da (ribosomal protein S6) present in the microsomal faction might be involved in this specific process.

The increase of the inhibition of LH/MIX-stimulated pregnenolone production with increasing doses of cycloheximide is significantly higher than the increase of the inhibition of unstimulated pregnenolone production. It has been found, however, that the relative effect of cycloheximide on translation-controlled synthesis of a single specific

protein under control and stimulated conditions is the same (Snoek et al., 1981; see also: Brooks, 1977; Rossow et al., 1979). Hence, the aberrant effects of cycloheximide on pregnenolone production may indicate the synthesis of more than one specific protein. Obviously, further experiments on the isolation of such proteins are required for further elucidation of the mechanism of hormonal control of the CSCC activity.

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