TRANSDUCING SYSTEMS IN THE HORMONAL REGULATION OF STEROIDOGENESIS IN RAT LEYDIG CELLS

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MECHANISMEN BETROKKEN BIJ DE SIGNAALOVERDRACHT BIJ DE HORMONALE REGULATIE VAN STEROIDOGENESE IN LEYDIG CELLEN VAN DE RAT

PROEFSCHRIFT

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CONTENTS

Other papers related to this thesis:

F.F.G. Rommerts, R. Molenaar, A.P.N. Themmen & H.J. van der Molen (1984) Regulation of steroidogenic activities in Leydig cells by LH and an LHRH agonist. In: Hormonal Control of the Hypothalamo-pituitary Gonadal Axis, K.W. McKerns & Z. Naor, eds, Plenum Press, New York, pp. 423-436.

A.P.N. Themmen, J.W. Hoogerbrugge, F.F.G. Rommerts & H.J. van der Molen (1984) Comparison of LH and LHRH agonist action on steroidogenesis in rat Leydig cells. Annals of the New York Academy of Sciences 438, 625-628.

A.P.N. Themmen, J.W. Hoogerbrugge, F.F.G. Rommerts & H.J. van der Molen (1985) A comparison of the mechanism of action of LH and an LHRH-agonist on steroidogenesis by immature rat Leydig cells. In: Recent Progress in Cellular Endocrinology of the Testis, J.M. Saez, M.G. Forest, A. Dazord & J. Bertrand, eds, Editions INSERM, Paris, pp. 299-305.

F.F.G. Rommerts, R. Molenaar, A.P.N. Themmen & H.J. van der Molen (1985) Hormonal regulation of testicular steroidogenesis via different transducing systems. In: Proceedings of the 10th European Symposium on Hormones and Cell Regulation, J. Nunez, J.E. Dumont & R.J.B. King, eds, Libbey & Co, London, in press.

A.P.N Themmen, J.W. Hoogerbrugge & F.F.G. Rommerts (1986) Transducing systems for the stimulation of steroid production in immature rat Leydig cells. In: Proceedings of the IVth International Testis Workshop on Molecular and Cellular Endocrinology of the Testis, M. Stefanini, ed., Elsevier, Amsterdam, in press.

F.F.G. Rommerts & A.P.N. Themmen (1986) LHRH, the role of LHRH (agonists) in the regulation of gonadal function. Acta Endocrinologica, in press.

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ABBREVIATIONS AND TRIVIAL NAMES

AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
ATP	adenosine 5'-trisphosphate
cAMP	adenosine cyclic-3',5'-monophosphate
cGMP	guanosine cyclic-3',5'-monophosphate
cholesterol	5-cholestene-3β-ol
CSCC	cholesterol side chain cleavage enzyme
cyanoketone	2α-cyano-17β-hydroxy-4,4',17α-trimethyl-5-androstene-3-one
CTP	cytosine 5'-trisphosphate
DAG	diacylglycerol
dbcAMP	N^6-2'-O-dibutyryl adenosine cyclic-3',5'-monophosphate
DDA	2',5'-dideoxyadenosine
EGF	epidermal growth factor
ER	endoplasmic reticulum
FSH	follicle stimulating hormone; follitropin
GTP	guanosine-5'-trisphosphate
GTPγs	guanosine-5'-(3-O-thio)-trisphosphate
hCG	human chorionic gonadotropin
IP	inositol-1-phosphate
IP_2	inositol-1,4-bisphosphate
IP_3	inositol-1,4,5-trisphosphate
LH	luteinizing hormone; lutropin
LHRH(-A)	luteinizing hormone releasing hormone (-agonist); luliberin
N_i	inhibitory guanine nucleotide binding protein
N_s	stimulatory guanine nucleotide binding protein
PA	4β-phorbol-13-monoacetate
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidylinositol-4,5-bisphosphate

PL-C	phospholipase C
PK-A	cAMP-dependent protein kinase
PK-C	Ca^{2+}/phospholipid dependent protein kinase
PMA	4β-phorbol-12-myristate-13-acetate
pregnenolone	5-pregnene-3β-ol-20-one
R_{II}	regulatory subunit of $PK\text{-}A_{II}$
R_i	receptor coupled to inhibition of adenylate cyclase
R_s	receptor coupled to stimulation of adenylate cyclase
SCP_2	sterol carrier protein 2
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate
SU-10603	7-chloro-3,4-dihydro-2(3-pyridyl)-1-(2H)-naphtalenone
testosterone	4-androstene-17β-ol-3-one
TFA	9-(tetrahydro-2-furyl) adenine
TFP	trifluoperazine
3β-HSD	3β-hydroxysteroid dehydrogenase
25-hydroxycholesterol	5-cholestene-3β,25-diol

CHAPTER 1 GENERAL INTRODUCTION

1.1 Introduction

In mammals, the testis can be divided into two specialized compartments, i.e. the tubular compartment and the interstitial compartment. In testicular tubules, the Sertoli cells form the lining of the inside of the tubular wall and they enclose the maturing germinal cells with many cytoplasmic extensions. Thus the male germinal cells are in intimate contact with the Sertoli cells. The development of germinal cells is dependent on FSH and testosterone, both acting on germinal cells via the Sertoli cell. In the interstitial compartment many different cell types are present such as Leydig cells, macrophages, fibroblasts and endothelial cells. The Leydig cells are the source of androgens which are important for spermatogenesis and development of the primary and secondary sex characteristics.

Steroidogenesis in the Leydig cell is mainly under the control of the pituitary hormone LH. However, it has been shown that other factors also can affect steroid production by Leydig cells, e.g. factors in interstitial fluid (Sharpe, 1984) and in medium from cultured Sertoli cells (Verhoeven & Cailleau, 1985) or from cultured testicular macrophages (Yee & Hutson, 1985). In addition, it has been found that LHRH-A (Hunter et al, 1982; appendix paper 2), arginine-vasopressin (Meidan & Hsueh, 1985), β-adrenergic agonists (Anakwe & Moger, 1984) and the atrial natriuretic factor (Pandey et al, 1985) can influence the activities of isolated Leydig cells. In addition to androgens Leydig cells can produce other factors such as prostaglandins (Molcho et al, 1984a,b), estradiol (Valladares & Payne, 1979; Rommerts et al, 1982a) and different peptides derived from pro-opiomelanocortin (Tsong et al, 1982; Margioris et al, 1983; Valenca & Negro-Vilar, 1986).

1.2 Scope of this thesis

Steroid production by the Leydig cell is subject to multiple regulation by many factors (see 1.1), which suggests that a complex for transduction of the external signals must be present in this cell. The factors that can regulate the activities of Leydig cells may utilize different second messenger systems, suggesting that such systems are present in the Leydig cell. Synergistic effects between the effect of LHRH and LH on

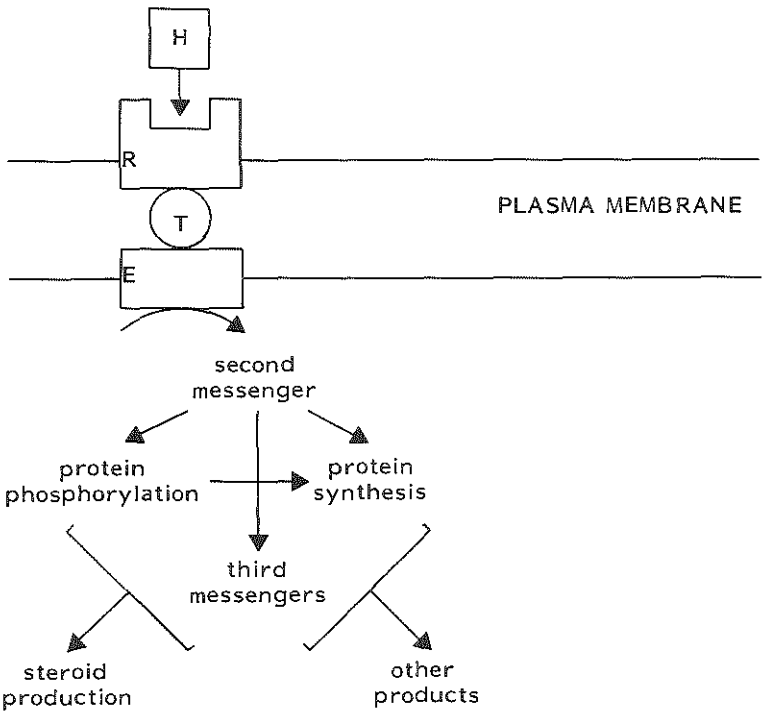

Figure 1.1 General scheme of transmembrane signalling. The hormone (H) binds to the receptor (R), thereby activating the transducer element (T). This results in an activation of the effector (E), which enhances the production of the intracellular second messenger. The second messenger can cause many effects in the cell e.g. stimulation of a small molecular weight "third messenger", stimulation of protein phosphorylation, or stimulation of protein synthesis. In the Leydig cell these (concomitant?) effects lead to increased productions of androgens and other compounds such as prostaglandins.

steroid production have been shown, suggesting that these hormones utilize independent pathways. However, several transducing systems are eventually coupled to the same target: regulation of steroid production via regulation of the activity of the cholesterol side chain cleavage enzyme. Others may be coupled to other functions of the Leydig cell such as growth and prostaglandin production.

The studies described in this thesis have mainly been concerned with the elucidation of several of the second messenger systems present in the Leydig cell and the regulation of steroidogenesis through pathways that involve cAMP and calcium ions as second messengers. A general, albeit simplified scheme for transmembrane signalling which result in stimulation of steroid production is given in figure 1.1.

To affect Leydig cell function specific stimulators (dbcAMP, phorbol esters etc.) or inhibitors (P-site agonists, diltiazem etc.) of the different second messenger systems were used in addition to physiological stimuli. The effects of these probes were measured by determining (1) the activity of adenylate cyclase through measurements of the intracellular levels of cAMP, (2) the activity of protein kinases by studying electrophoresis patterns of proteins labelled with $^{32}PO_4$, (3) stimulation of de novo protein synthesis by studying the electrophoretic patterns of proteins labelled with ^{35}S-methionine and (4) the activity of the cholesterol side chain cleavage enzyme by measuring the production of pregnenolone from endogenous precursors.

The results of these studies are described in detail in chapters 3-6 and in the appendix papers 1, 2, 4 and the appendix chapter. Results of studies on the involvement of cAMP and calcium as second messengers in LH action are described in chapter 4. The mechanism of action of LHRH is discussed in chapter 5 and data on the presence of GTP-binding proteins N_s and N_i in Leydig cells are given in chapter 6. In chapter 7 an integrated model of the regulation of properties of Leydig cell function is discussed with special emphasis on the conclusions derived from the results in the preceding chapters are summarized.

CHAPTER 2 TRANSDUCING SYSTEMS

2.1 General introduction

Many protein hormones acting on cells, exert their effects via an interaction with proteins (receptors) on or in the plasma membrane, which have a high affinity for the hormone. The receptor activates other proteins in the cell membrane, eventually resulting in the production of one or more second messengers inside the cell. This process is called "transduction" or "transmembrane signalling" of hormonal signals, and the different hormone-receptor-transducer-second-messenger systems are also called "second messenger systems". (See also figure 1.1).

In this chapter background information concerning the different transducing systems or transmembrane signalling systems which are discussed in this thesis will be provided. This section is not intended to give a comprehensive review of all transducing systems, and only those systems which are important for Leydig cell functions as described in this thesis will be discussed. Discussion will include the systems that use cAMP, IP_3, calcium or diacylglycerol as second or third messenger. Other systems that are present in the Leydig cell such as the tyrosine kinase system (coupled to the EGF receptor), the guanylate cyclase system (coupled to the atrial natriuretic factor receptor) will not be discussed.

In the following subsections we will consider the different second messenger systems, their most important matching receptors, and their mechanism of coupling to production of the intracellular messenger. The concluding paragraph presents a recent idea for a unified theory of transmembrane signalling.

2.2 Transducing systems involving cAMP

2.2.1 introduction

The role of cAMP as an intracellular messenger was discovered by Sutherland and colleagues who studied the stimulatory effects of glucagon and epinephrine on phosphorylase activity, and reported in 1957:

> "The response to the hormones in liver homogenates was separated in two phases: first, the formation of an active factor in the particulate fractions in the presence of hormones and, second, the stimulation by the factor of liver phosphorylase formation in supernatant fractions of homogenates in which the hormones themselves had no effect", and "The factor was heat-stable, dialyzable, and was purified considerably by chromatography on anion and cation exchange resins" (Rall et al, 1957).

Five years later a partly purified preparation of active adenylate cyclase was described (Sutherland et al, 1962). In the ensuing decades considerable effort has been spent on the investigation of the hormonal regulation of adenylate cyclase (reviews: Ross & Gilman, 1980; Schramm & Selinger, 1984). These studies resulted in a now generally accepted cascade of events which can be summarized as follows.

There is a multitude of receptors that affect cAMP formation. These receptors (R) can be classified into two sub-types: R_s receptors, which cause increased cAMP formation by stimulating adenylate cyclase, and R_i receptors, which cause decreased cAMP levels by inhibiting adenylate cyclase (Birnbaumer et al, 1985). Receptors for glucagon, ACTH, LH, FSH, TSH, adenosine (A_2-receptor) and for β-cathecholamines are of the R_s type, whereas the muscarinic (acetylcholine), α_2-cathecholamine, adenosine (A_1-receptor) and the somatostatin receptor are of the R_i type. Both types of receptor can be present in the same cell e.g. Sertoli cells contain the R_s for FSH (Fletcher & Reichert, 1984), glucagon (Eikvar et al, 1985) and β-adrenergic agonists (Kierzenbaum et al, 1985), and

the R_i for adenosine (A_1; Monaco et al, 1984), in addition to tyrosine kinase receptors such as the insulin receptor (Oonk et al, 1986).

2.2.2 GTP-binding proteins: N_s and N_i

When it was accepted that the interaction between extracellular hormones and membrane receptors could affect the activity of adenylate cyclase on the inside of the cell membrane, much effort was invested to elucidate the molecular mechanism of the coupling of R_s and R_i to this enzyme.

A central role of GTP-binding proteins (N proteins) in hormonal regulation of adenylate cyclase was first suggested by Rodbell and colleagues, who demonstrated that GTP was required for glucagon activation of the enzyme in plasma membranes from liver (Rodbell et al, 1971b; Harwood et al, 1973). In addition, they showed that GTP enhanced the rate of dissociation of radiolabelled glucagon from its receptor binding sites (Rodbell et al, 1971a). This mechanism involving the GTP-binding proteins (N proteins) has been clarified in detail by investigations on the β-adrenergic receptor system and on erythrocyte membranes (Gill & Meren, 1978; Cerione et al, 1985; reviews: Birnbaumer et al, 1985; Northrup, 1985).

The N protein coupled to R_i (N_i) differs from the N protein coupled to R_s (N_s), although these proteins have a similar subunit structure. A model of the structure and function of N_s and N_i is given in figure 2.1. N_s consists of a GTP-binding subunit α_s (42-52 kDa), a β-subunit (35 kDa) and a small γ-subunit (ca. 5 kDa). N_i consists of an α_i-subunit (41 kDa), the same β-subunit as N_s, and a small γ-subunit which may be the same as in N_s. The functional importance of the γ-subunits is not known. The N proteins not only bind GTP, but also act as GTPases. Upon binding of the hormone to the receptor (R_s or R_i, the mechanism of the N proteins is symmetrical), the α-subunit of the intact, inactive ($\alpha\beta\gamma$) N protein binds GTP, and the α-GTP and $\beta\gamma$-unit dissociate. The activated α_s-GTP associates with the adenylate cyclase thereby activating the enzyme,

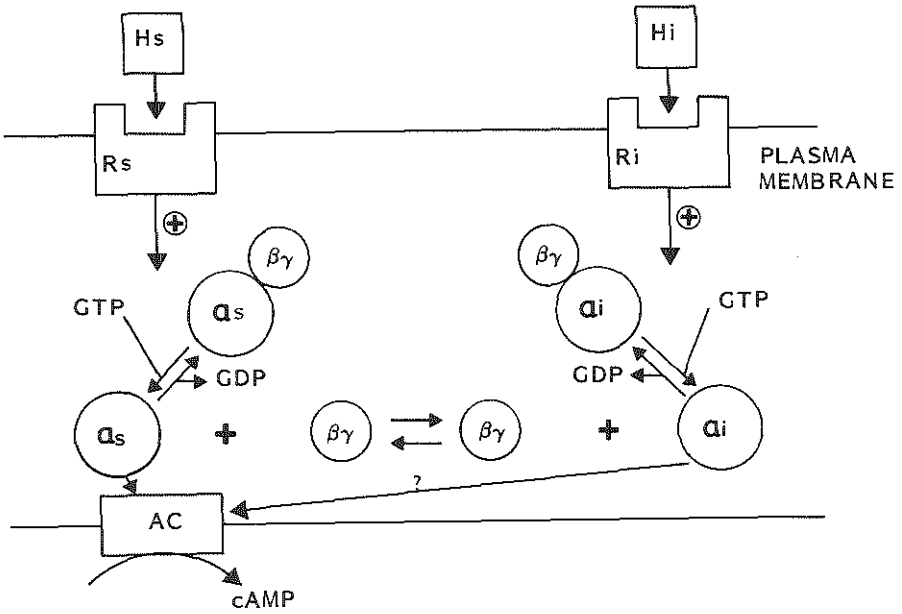

Figure 2.1 Schematic representation of the involvement of N_s (consisting of $\alpha_s\beta\gamma$) and N_i (consisting of $\alpha_i\beta\gamma$) in signal transduction. For explanation see text. AC: adenylate cyclase; H_s, H_i: hormones, binding to R_s or R_i respectively.

which results in an increase production of cAMP. Because the $\beta\gamma$-units of N_s and N_i are thought to be identical, a single pool of $\beta\gamma$-units may be present in membranes. Activation of N_i may therefore inhibit cAMP formation in two ways: either via direct binding of the α-subunit of N_i to adenylate cyclase, although this could not be confirmed with in vitro studies, or via enlargement of the pool of $\beta\gamma$-units, which may bind to α_s-subunits leading to deactivation of N_s. Both α_s-GTP and α_i-GTP are inactivated by a GTPase dependent association to the $\beta\gamma$-subunit giving rise to the intact, inactive N protein. It has been shown that the affinity of the receptor is affected by N_s. Association of N_s with the β-adrenergic receptor results in a high affinity state of the receptor. During activation of adenylate cyclase by α_s, the receptor changes to a low affinity state, to be reactivated by the GTPase dependent association of

N_s (DeLean et al, 1980; Limbird et al, 1980; Stadel et al, 1980).

Of great interest is the ability of two bacterial toxins, cholera toxin and pertussis toxin, to catalyze the transfer of an ADP-ribosyl-group from NAD to the α-subunit of the N proteins both in vivo and in vitro. Cholera toxin ADP-ribosylates α_s (Gill & Meren, 1978), whereas pertussis toxin acts on α_i (Katada et al, 1982). The α-subunits can be labelled, through incubation with the appropriate toxin in the presence of ^{32}P-labelled NAD, and can be subsequently detected after separation on SDS-PAGE (see appendix paper 5). Toxin treatment of the N proteins has a profound effect on their activity (review: Gilman, 1984). ADP-ribosylation of α_s by cholera toxin inhibits the GTPase activity, prevents the reassociation of α_s to the $\beta\gamma$-subunit which results in a permanent activation of α_s and adenylate cyclase. Pertussis toxin treatment of α_i also inhibits the GTPase activity, but this results in an increased affinity of α_i for $\beta\gamma$. The low concentration of free α_i causes a stimulation or abolishment of inhibition of adenylate cyclase in many systems. Pertussis and cholera toxin have proven to be valuable tools in the study of the involvement of N_s or N_i in the action of hormones.

2.2.3 adenylate cyclase and cAMP-dependent protein kinase

Little is known about the adenylate cyclase enzyme. The molecular mass is ca. 150 kDa, and it catalyzes the production of cAMP from MgATP or MnATP substrates (Schlegel et al, 1979). The enzyme can be obtained in a "resolved state", i.e. not purified, but free from N_s or N_i (Ross et al, 1978). Using this resolved preparation it has been found in a reconstituted system with the β-adrenergic receptor that N_s and N_i are both necessary to obtain a maximal stimulation by agonists (Cerione et al, 1985).

The only known role in eukaryotes of the product of adenylate cyclase, cAMP, is activation of cAMP-dependent protein kinase (PK-A). The two types of PK-A (PK-A_I and

PK-A_{II}), have been shown to be similar in size. They have a similar subunit composition and mechanism of activation, but differ in the characteristics of their regulatory subunits (review: Lohmann & Walter, 1984). The two regulatory subunits, designated R_I and R_{II}, have molecular weights of 47 and 54 kDa respectively. The catalytic subunit (C; 40 kDa) of both enzymes is identical. Both kinases are activated by binding of cAMP to R, resulting in a dissociation of the holoenzyme as indicated in the following scheme.

$$R_2C_2 \text{ (inactive)} + 4 \text{ cAMP} \rightleftharpoons R_2\text{-(cAMP)}_4 + 2 \text{ C (active)}$$

The activity is not only regulated by cAMP, but may also be affected by phosphorylation of the regulatory subunits. PK-A_{II} undergoes autophosphorylation by the catalytic subunit, and it has been shown that this phosphorylation results in a slower reassociation of the R and C subunits (Rangel-Aldao & Rosen, 1977). Both R_I and R_{II} can be phosphorylated also by other kinases, but the significance of this phosphorylation in the regulation of PK-A is not clear (Lohmann & Walter, 1984).

Many different proteins can serve as substrates of PK-A, but it is beyond the scope of this chapter to discuss all these proteins. It has been found that PK-A is involved in many different expressions of cell function, such as metabolic activity (phosphorylation of liver pyruvate kinase (Engstrom, 1980)), protein synthesis (ribosomal protein S6 (Traugh, 1981)) and cell shape (cytoskeleton proteins (Osawa & Hall, 1985)). Specific phosphoproteins may play a major role depending on the function of the cell, e.g. phosphorylase in liver.

2.3 Transducing system involving phosphoinositide metabolism

2.3.1 introduction

During the last four years evidence has accumulated that in

addition to cAMP-dependent signalling systems, another important system involving phospholipid breakdown is present in all cells. Hokin & Hokin (1953, 1954) reported that stimulation of pancreas slices with acetylcholine resulted in an increase in turnover of phosphatidylinositol (PI). This observation has been repeated with many different cell types for many different agonists, and phospholipid breakdown is now generally accepted as a versatile receptor-activated signalling system, that stimulates different activities such as intracellular calcium levels, protein kinase C and metabolism of arachidonic acid. In this subsection only the main properties of this system will be discussed (for reviews: Berridge, 1984; Takai et al, 1984; Downes & Michell, 1985; Majerus et al, 1985).

2.3.2 the phosphoinositide cycle

Many different agonists stimulate PI-breakdown in cells. Adrenergic agonists (through the α_2-receptor), vasopressin (V_1-receptor), serotonin (5-HT_1-receptor) and acetylcholine (muscarinic receptor) are all coupled through this system to stimulation of intracellular calcium levels (review: Berridge,

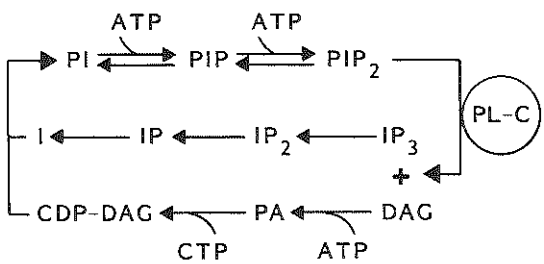

Figure 2.2 The reactions of the PI-system. All reactions take place in the plasma membrane. For explanation see text. Abbreviations: IP_3: inositol trisphosphate; IP_2: inositol bisphosphate; IP: inositol phosphate; I: inositol; PI: phosphatidyl inositol; PIP: phosphatidyl inositolmonophosphate; PIP_2: phosphatidyl inositolbisphosphate; PL-C: phospholipase C: CDP-DAG: cytosine bisphosphate-diacylglycerol; PA: phosphatidic acid.

1982). A general outline of the PI-system is given in figures 2.2 and 2.3.

The major step in the PI system is the receptor-mediated breakdown of phosphatidylinositol bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG), through activation of a phospholipase C. IP_3 can stimulate the release of calcium from intracellular stores and DAG can either activate protein kinase C, or DAG can be cleaved to release arachidonic acid which can serve as a substrate in the formation of several arachidonic acid metabolites. These three pathways of the PI-system will be discussed below.

There is increasing evidence that the effect of the hormone-receptor complex on PL-C is mediated by a GTP-binding protein. With isolated membrane preparations it can be shown that GTP stimulates hormone-dependent IP_3 production (Litosch et al, 1985), and many studies show that treatment of cells with per-

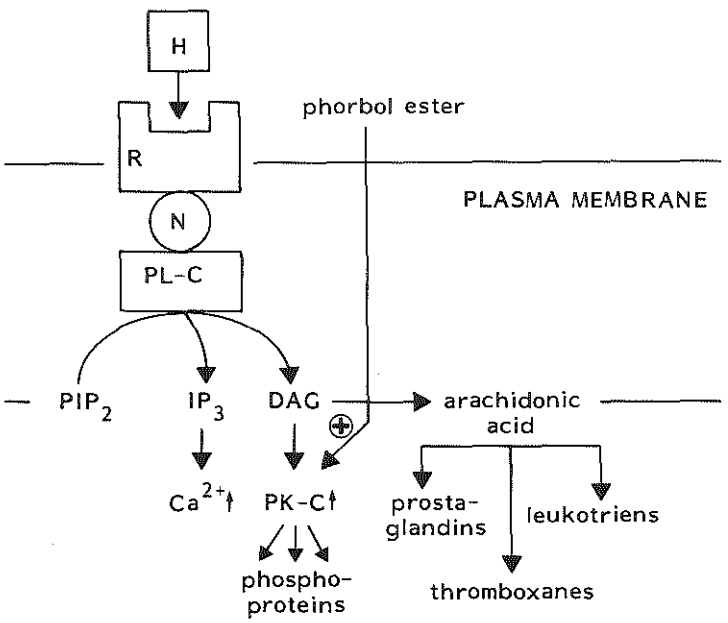

Figure 2.3 General outline of the PI-system, showing the three pathways leading to elevation of intracellular free calcium levels, phosphorylation of proteins and production of arachidonic acid metabolites.

tussis toxin inhibits the subsequent hormone-stimulated PIP_2 breakdown (Evans et al, 1985; Uhing et al, 1986). The nature of this GTP-binding protein is unknown, but awaiting further characterization it has been named Np.

Regeneration of the phosphoinositides occurs as follows. Cellular IP_3 will be metabolized rapidly to IP_2, IP and finally to inositol. DAG is activated using CTP and reacts with inositol to form PI. PI is phosphorylated to form PIP and PIP_2, closing the cycle (figure 2.2). The latter two reactions can be catalyzed in vitro by two tyrosine kinase oncogene products (src and ros), indicating that growth factors may modulate the PI system via activation of their receptor-tyrosine kinases.

2.3.3 the three messengers

The first pathway, the production of IP_3, links the PI system to the control of intracellular calcium levels. Using permeabilized pancreatic acinar cells (Streb et al, 1983) and hepatocytes (Burgess et al, 1984) it was shown that IP_3 can release calcium from the endoplasmic reticulum. This effect is IP_3 specific with other inositol phosphates being inactive (Berridge, 1984). The role of increased intracellular levels of calcium will be discussed below (see subsection 3.4).

The second pathway, via the production of DAG, is directly related to protein phosphorylation. DAG can activate calcium/phospholipid-dependent protein kinase (PK-C), which was first described by Nishizuka (review: Takai et al, 1984). Both calcium and DAG are required for activation of PK-C in vitro. DAG can increase the sensitivity of PK-C to calcium as much as 1,000 fold, and the inactive enzyme can be completely activated by DAG even at cytosolic calcium concentrations present in resting cells (Takai et al, 1981). Hence, calcium is an essential requirement for the enzyme, but not the key regulator of PK-C activity in vivo.

The discovery that PK-C can be activated directly by tumor-promoting phorbol esters, provided a tool to activate PK-C in intact cells without the possible interference of other

second messenger systems (Castagna et al, 1982; Niedel et al, 1983). In experiments using phorbol esters, or measuring the DAG-stimulated activity of the isolated enzyme, it was shown that PK-C is an ubiquitous enzyme, capable of phosphorylation of proteins associated with many cell functions, e.g. membrane proteins (Kiss & Luo, 1986), ribosomal protein S6 (Trevillyan et al, 1984) and cholesterol side chain cleavage enzyme (Vilgrain et al, 1984). PK-C also phosphorylates N_i thereby suppressing its activity (Katada et al, 1985), and it has been shown that phorbol ester treatment of cells can inhibit N_s function (Mukhopadhyay & Schumacher, 1985). These results suggest that there may be a regulatory link between the cAMP- and the PI-system.

The third pathway of the PI system involves the stimulation of arachidonic acid metabolism. Phosphoinositides have been shown to be very rich in 1-stearoyl-2-arachidonyl. After the breakdown of PIP_2 the DAG can be further metabolized by 1,2-DAG-lipase (Lenstra et al, 1984). This enzyme sequentially removes stearic acid from position 1 and arachidonic acid from position 2 of DAG (Prescott & Majerus, 1983). The released arachidonic acid can serve as a substrate for the formation of different bioactive metabolites including prostaglandins, leukotrienes and thromboxanes. These metabolites can influence receptors on the cell of origin or on nearby cells, and in turn may influence the PI system again (Majerus et al, 1984).

The considerations given above show that the PI-system is a versatile system that affects many different cell functions through the three different intermediates: calcium, DAG and arachidonic acid metabolites. A general model of the PI-system is given in figure 2.3. It may well depend on the specific target cell which of these pathways will be most important in that particular cell. Finally, there are many links between the cAMP and PI system, e.g. PK-C may act on N proteins, PK-A and PK-C can phosphorylate identical proteins and prostaglandin can stimulate adenylate cyclase. These examples show that the two second messenger systems could be intimately involved with one another.

2.4 Role of calcium as a messenger

2.4.1 introduction

Like cAMP, calcium is ubiquitously present and may be involved in many aspects of cell regulation (reviews: Rasmussen & Barrett, 1984; Huggins & England, 1985). Many hormones increase intracellular calcium levels in their target cells and their physiological action is abolished when these increases are inhibited, or when the actions of calcium ions are prevented with specific inhibitors, such as calmodulin inhibitors. Effects of hormones that depend on the presence of calcium ions include: the effect of platelet-derived growth factor and epidermal growth factor on human fibroblasts (Moolenaar et al, 1984), thyrotropin releasing hormone acting on anterior pituitary cells (Ozawa & Kimura, 1982), LH and LHRH-A acting on Leydig cells (Sullivan & Cooke, 1986) and noradrenaline, vasopressin or angiotensin acting on liver cells (Mauger et al, 1984). The calcium ions involved in the increase of the intracellular calcium concentration can either originate from outside the cell, or can be liberated from intracellular stores. Both sources are under hormonal control, and are important in the increase of the intracellular free calcium concentration. In this subsection the regulation of the intracellular calcium concentration and the effects of calcium in the cell will be discussed.

2.4.2 regulation of the intracellular calcium concentration

The intracellular concentration of free calcium in the cytoplasm is extremely low (in the micromolar range) as compared to the extracellular calcium concentration (in the milimolar range (Campbell, 1983)), resulting in a steep calcium-gradient accross the plasma membrane. Influx of calcium through calcium channels may occur from three sources: from the extracellular space, and from the endoplasmic reticulum (ER) and the mitochondrion, which accumulate calcium against a steep gra-

dient. The mitochondrion is not considered to be involved in the hormone-stimulated increase in intracellular calcium (see subsection 2.3.3). Because high intracellular calcium is cytotoxic (Rasmussen & Barrett, 1984), the cell has an elaborate system of pumps that actively decrease the cytoplasmic calcium: Ca^{2+}-ATP-ases on the plasma membrane, mitochondrion and ER, and Na^{+}-Ca^{2+}-exchangers in the plasma membrane. The different transport systems are outlined in figure 2.4.

It has been found that hormones can regulate cytoplasmic calcium 1) through activation of PIP_2 metabolism, resulting in IP_3 formation that liberates calcium from the ER (see subsection 2.3), 2) through voltage-dependent calcium channels in the plasma membrane and 3) through release initiated by depola-

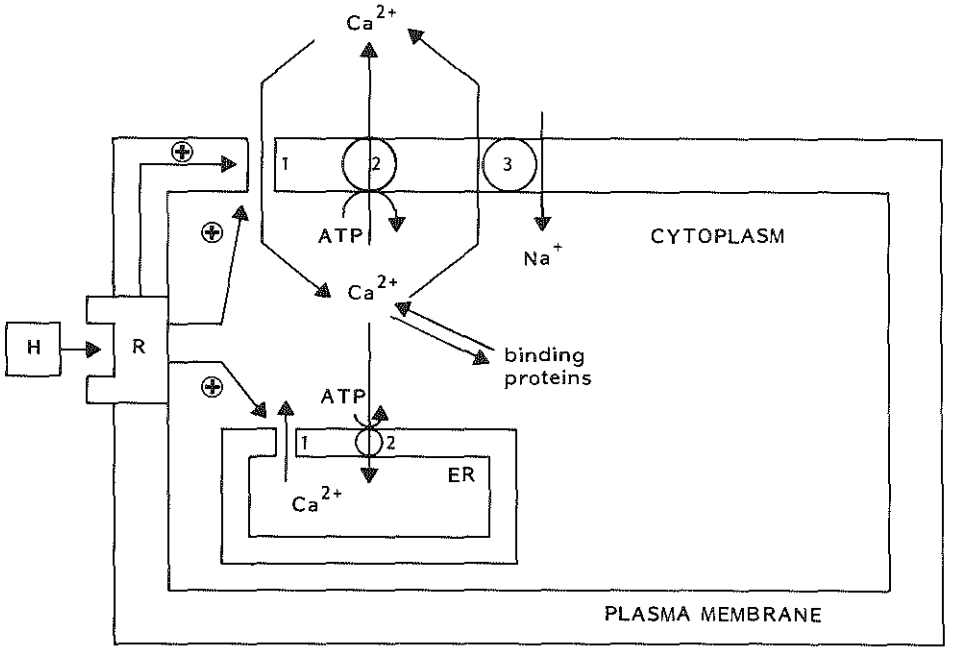

Figure 2.4 Different calcium transport systems in the cell. The mitochondrial and bound calcium pools are not shown. Three different calcium transport systems are shown: 1) calcium channel; 2) Ca^{2+}-ATPase-coupled calcium transporter (Ca^{2+}-ATPase); 3) Na^{+}-Ca^{2+}-antiporter.

rization from internal stores. The latter process is of major significance in electrically excitable tissues, and will not be discussed. There is some evidence for the regulation of intracellular calcium by voltage-dependent calcium channels in hormonally regulated cells. Cloned cells from the anterior pituitary have been shown to contain voltage-dependent calcium channels, and thyrotropin-releasing hormone stimulates the entry of calcium by changing some properties in membrane polarization in GH_3 cells (Ozawa & Kimura, 1979). This effect of thyrotropin-releasing hormone could be blocked by the calcium channel antagonist verapamil (Ozawa & Kimura, 1982). Furthermore, it has been shown in isolated rat liver cells, that noradrenalin, vasopressin and angiotensin increase calcium influx by opening a common pool of calcium channels (Mauger et al, 1984). There is increasing evidence that the coupling of the receptor to the calcium channel may also be mediated by a GTP-binding protein, although the nature of this N protein is not known (Gomperts, 1983; Koch et al, 1985; Holz IV et al, 1986).

2.4.3 functional effects of calcium in the cell

Functional effects of an increase in intracellular calcium are always mediated by calcium-binding proteins. There are two classes of these proteins: 1) true calcium receptor proteins such as calmodulin, parvalbumin and troponin C, which undergo a conformational change upon calcium binding, and subsequently interact with enzymes dependent on these proteins for their activity, and 2) enzymes directly regulated by calcium, such as Ca^{2+}-activated protease or protein kinase C.

Calmodulin is the most extensively described calcium binding protein (reviews: Klee et al, 1980; Veigl et al, 1984). Its molecular mass is ca. 16 kDa and there are four sites for binding calcium. Upon binding of two to three calcium ions the protein changes its conformation, becomes active, and binds to the calmodulin-target proteins. Many phosphorylation-dephosphorylation systems such as glycogen phosphorylase are regulated

by calmodulin. In addition, calmodulin is very important in the regulation of the cytoskeleton, because it interacts with molecules such as caldesmon and spectrin, and can also activate tubulin kinase. Calmodulin can stimulate cAMP hydrolysis through activation of a calmodulin dependent phosphodiesterase (Erneux et al, 1985), and it may serve as one of the links between the calcium- and the cAMP-systems.

For the second class of proteins the regulatory role of calcium is less clear. Many of these enzymes (e.g. Ca^{2+}-activated protease) have been shown to be regulated by calcium in vitro, but this requires calcium concentrations in the mMolar range, which are not attained in the intact cell.

In conclusion, available evidence indicates that increases in intracellular calcium may play a role in many different aspects of cell regulation ranging from modulations of the cAMP system, through activation of protein kinases to changes in cytoskeleton and intermediary metabolism.

2.5 Concluding remarks

GTP-binding proteins appear to play an important role in the initial action of the hormones on all second messenger systems discussed in this chapter. N_s and N_i are involved in the regulation of adenylate cyclase, and effects of GTP and pertussis toxin on the hormone-regulated calcium channels and the PI system have been observed. Transducin, the protein that couples the effect of light-activated rhodopsin to cGMP-phosphodiesterase is also an N protein (Fung, 1985). Recently an N protein with unknown function has been purified from brain (N_o) (Sternweis & Robishaw, 1984). There are indications that some insulin effects are mediated by an N protein (N_{ins}) (Houslay, 1985).

These observations suggest that there may be a family of GTP-binding proteins which mediate the transduction of signals of hormone-receptor binding to various intracellular events. Based on these observations, Rodbell (1985) has proposed a theory on hormone action involving programmable messengers as

outlined in figure 2.5. A hormone interacting with its receptor stimulates the release of an activated α-subunit. This α-subunit can be covalently modified in different ways by a modifier M (phosphorylation, methylation, sulphation), each modification directing the α-subunit to activate another effector E (adenylate cyclase, phospholipase C). The modification of the α-subunits can be considered as programming, hence the term "programmable messengers". The effectors emit a signal S (cAMP, calcium) that cause the final response R. This theory has yet to be verified experimentally, although it has been shown with reconstituted systems, as well as in intact cells, that binding of hormone to the receptor can result in the release of the α-subunit into the cytoplasm is feasible (Dominguez et al, 1985; Rodbell, 1985; Sternweis, 1986).

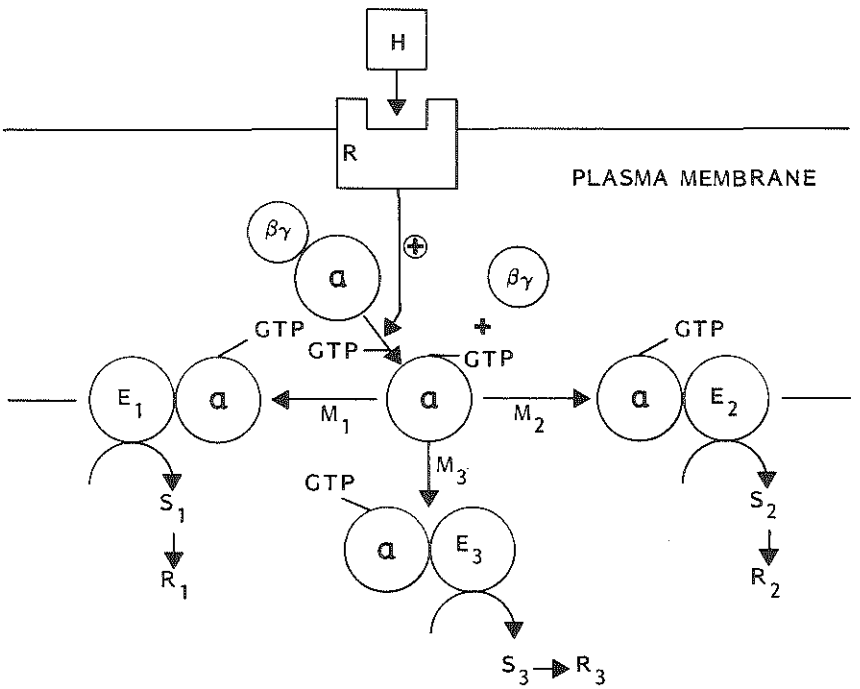

Figure 2.5 Proposed model of "programmable messengers". (Adapted from Rodbell (1985)). For explanation see text.

CHAPTER 3 METHODS

3.1 Introduction

This chapter concerns those methods which are of special interest for the work presented in this thesis, and which are not described in detail in the appendix papers. All other relevant materials and methods have been described previously and can be found in the appendix papers.

3.2 Isolation and characterization of Leydig cells from immature rats

Interstitial cells obtained from immature (21-24 days old) rats were used for most of the studies described in this thesis. Preliminary results had shown that these cells responded very well to LH and LHRH-A, and could be kept in culture for 72 h without considerable loss of steroid response. Leydig cells from immature testes were obtained in a high yield (90%) and with an acceptable (40-50%) purity as follows: after collagenase dispersion of the decapsulated testes, the cells in the supernatant were washed and allowed to attach to the plastic surface of a culture dish in Eagle's minimal essential medium with Earle's salts and non-essential amino acids (MEM) containing 1% (v/v) foetal calf serum (32°C, 5% CO_2 in air). After one hour of culture the floating cells were removed by washing and the attached cells were kept in MEM with 1% (w/v) bovine serum albumin (Rommerts et al, 1985). In this preparation 40-50% of the cells were positive for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity (for staining method see Rommerts et al, 1985), which was used to estimate the number of Leydig cells (see appendix paper 3). To investigate the effect of hormones on steroid production the cells were incubated with inhibitors of pregnenolone metabolism (cyanoketone and SU-10603; see appendix paper 2), and pregnenolone production was measured in the culture medium by radioimmunoassay. Under these

conditions pregnenolone production was a good parameter for measuring cholesterol side chain cleavage (CSCC) activity, since pregnenolone is the direct product of the cleavage of cholesterol. Measurement of testosterone may underestimate the steroidogenic activity because only a small fraction of pregnenolone is converted to testosterone in Leydig cells of immature rats.

Interstitial cell preparations from immature rats also contain other cells in addition to Leydig cells, and it could not be excluded a priori that these non-Leydig cells might contribute to steroid production. In this regard we have investigated the steroidogenic properties of these cells after further fractionation of the cell preparation on a 30-60% Percoll density gradient. After attachment of the cells from different fractions to petri dishes, the potential CSCC activity and the hormonal regulation of the enzyme activity were determined by measuring the 25-hydroxycholesterol-, the LH-dependent and the basal pregnenolone production. The presence of 3β-HSD activity was used as a marker for endoplasmic reticular steroidogenic enzymes. As shown in figure 2 in appendix paper 3, the ratio of the different activities (LH/3β-HSD or 25-OH/3β-HSD) in the fractions was approximately constant. These results indicate that the LH-stimulated CSCC activity is present only in 3β-HSD-positive cells, and that the 3β-HSD-negative cells do not contribute to the steroidogenic response. Using this Percoll density gradient centrifugation technique a preparation containing 90% Leydig cells could be obtained (see: appendix paper 3). This preparation was used in the experiments on protein synthesis (chapter 5 and appendix paper 4).

3.3 Incubation conditions

The stimulatory effect of LH on pregnenolone production is very rapid. Within 2 minutes following the addition of a maximal stimulatory dose of the hormone, the first effects on steroid production can be detected, and after 20 minutes the stimulation is maximal (Rommerts et al, 1982b). In contrast,

stimulation of steroid production by LHRH-A evolves much slower (Hunter et al, 1982). This difference in kinetics of steroid response might reflect a difference in the mechanism of action of these hormones. Hence, we have determined the rate of pregnenolone production at different time-points after stimulation of Leydig cells with LH (100 ng/ml), LHRH-A (40 nM), and a dose of LH (0.1 ng/ml) which stimulated steroid production to the same extent as 40 nM LHRH-A (figure 3.1). The results show that the effect of LHRH-A occurs more slowly than that of LH (20 minutes for first detection), and that the maximal effect is reached after 3 h. Stimulation of steroid production with 100 ng/ml LH starts a few minutes after the addition of LH, and is maximal within 30 minutes. The results also show that the kinetics of the effect of 0.1 ng/ml LH are very similar to the kinetics of 40 nM LHRH-A, although after 4 h the rate of preg-

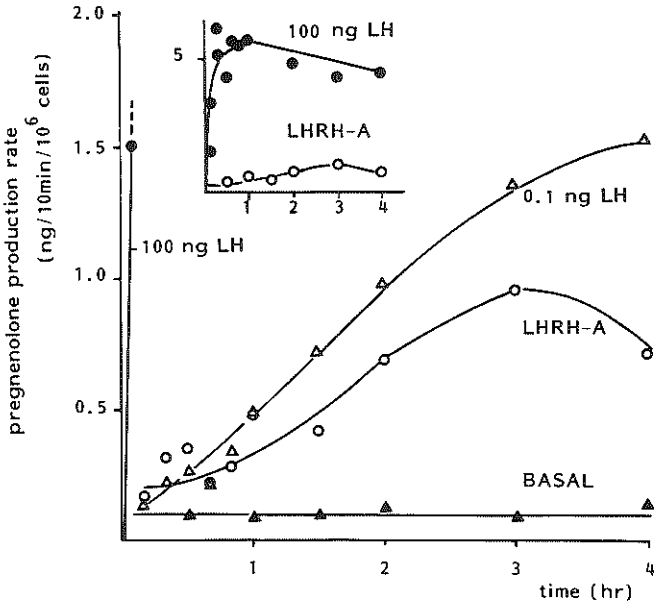

Figure 3.1 Kinetics of LH (inset) and LHRH-A action on pregnenolone production by Leydig cells isolated from immature rats. Pregnenolone production per 10 min was calculated from the concentrations of pregnenolone in culture media from cells incubated during different periods. Mean results from triplicate determinations are shown.

nenolone production in the presence of LHRH-A declines, whereas the stimulation with 0.1 ng/ml LH still has not reached its maximal value. These data indicate that, depending on the concentration of LH, LHRH-A and LH have similar effects. This may suggest an analogous mechanism of action of LH and LHRH-A at concentrations with a low steroidogenic potency. However, the experiments concerning the involvement of calcium (chapter 4 and 5), show that the mechanism of LH and LHRH-A is different at all concentrations of LH.

The results depicted in figure 3.1 show that the effects of LHRH-A on steroid production are maximal after 3 h. Consequently, for all investigations concerning LHRH-A action 3 h incubations were used.

3.4 Protein phosphorylation

To determine patterns of protein phosphorylation in intact cells, 10^6 cells, attached to a plastic culture dish (35 mm diameter), were incubated with 200 μCi/ml $^{32}PO_4$ (carrier free) for 3 h in Krebs-Ringer buffer without phosphate but containing 0.2% (w/v) glucose and 0.1% (w/v) bovine serum albumin. The proteins were extracted and separated using polyacrylamide gradient (8-15%) gel electrophoresis in the presence of SDS (SDS-PAGE) as described by Bakker et al (1981). The labelled proteins were then visualized by autoradiography. Using this method a high background radiation which interfered with the assessment of the phosphorylated proteins was observed. This background labelling could not be suppressed, although different approaches were used: e.g. extraction of RNA and DNA prior to electrophoresis; decrease of the labelling period; different extraction methods of proteins using trypsin, SDS-containing lysis buffer and trichloroacetic acid; variation of the amount of $^{32}PO_4$ present during the incubation; repeated precipitation and dissolving of the proteins with 10% trichloroacetic acid and an SDS-containing buffer, respectively; labelling of the proteins and washing of the protein pellet in the presence of trisphosphate, to remove labelled polyphosphates which may be

present; extraction of phospholipids before SDS-PAGE and lastly, extensive washing of the gel in the presence of unlabelled phosphate. These approaches did not improve the autoradiographs significantly. The same difficulties were encountered in phosphorylation studies with Leydig cells from mature rats. However, following subfractionation of the cells better results were obtained. Subfractionation of Leydig cells from immature rats proved to be difficult because the small diameter of the cells prevented cell disruption in commercially available Dounce glass-glass homogenizers (clearance: 0.025-0.030 mm). Therefore, to solve the problem of the high background radiation in phosphorylation experiments with immature rat Leydig cells, a glass-glass homogenizer with a smaller clearance, that will disrupt small cells, should be used.

To study the possible effects of protein kinases on endogenous substrates in total Leydig cell homogenates or in a 25,000xg supernatant from Leydig tumour cells the following method was used (adapted from de Jonge (1976)). Homogenates (40-100 μg protein) were incubated for 2 min at 30°C in 25 mM 2-glycerolphosphate, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4 in the presence of 5 μCi $(\gamma)^{32}$P-ATP with 0.01 mM unlabelled ATP, with one of the following additions to activate a particular kinase: cAMP-dependent protein kinase: 5 μM cAMP; cGMP-dependent protein kinase: 1 μM cGMP; calcium-dependent protein kinase(s): 2 mM $CaCl_2$; Ca^{2+}-calmodulin-dependent protein kinase: 2 mM $CaCl_2$ with 250 μg/ml calmodulin (partly purified from rat brain; kind gift from Dr. de Jonge); calcium/phospholipid-dependent, protein kinase (PK-C): 2.5 mM $CaCl_2$, 0.25 μg/ml phosphatidylserine (in suspension), 2 μg/ml PMA. The volume was adjusted to 50 μl with H_2O, the reaction was started by adding $(\gamma)^{32}$P-ATP, and terminated by adding SDS-PAGE sample buffer (Bakker et al, 1981). A part of the sample was separated on SDS-PAGE (8-15%), and the gel was prepared for autoradiography. After autoradiography results were obtained which did not have the high background problem such as encountered with the phosphorylation method used for intact cells.

3.5 Western blotting and photo-affinity labelling of R_{II}

For the identification of the regulatory subunit of type II cAMP-dependent protein kinase (R_{II}) in the 25,000xg supernatant of a tumour Leydig homogenate two approaches were chosen: a Western-type blotting experiment using a specific polyclonal antibody against R_{II} (kindly provided by Drs. Walter and Lohmann, Departments of Physiological Chemistry and Medicine, University of Wurzburg) and a photoaffinity-labelling method with (^{3}H)8-azido-cAMP.

The Western-blotting experiment was carried out as described by Ratoosh & Richards (1985), with some modifications. The proteins were separated on SDS-PAGE (10% polyacrylamide) and electrophoretically transferred to a nitrocellulose filter (Burnette, 1981). The filter was incubated overnight with phosphate buffered saline (PBS), 5% (w/v) BSA. The filter was subsequently incubated with the anti-R_{II} serum (diluted 1:500 in PBS, 0.1% (w/v) gelatin, 5% (w/v) BSA, 0.05% (w/v) Tween, 0.3% (w/v) Triton X-100) for 5 h at room temperature and overnight at 8°C. After 4 washes with wash-buffer (PBS, 0.05% (w/v) Tween, 0.3% (w/v) Triton X-100), the filter was incubated for 2 h at room temperature with ^{125}I-labelled Staphylococcal protein A (150,000 cpm/ml in PBS-gelatin-BSA-Tween-Triton buffer). After washing, the filter was dried (30 min at 60°C) and placed with Kodak SB-5 X-ray film.

R_{II} was labelled with (^{3}H)8-azido-cAMP using a photoaffinity-labelling method (Richards et al, 1983; Brinkmann et al, 1986). 70 µl of the 25,000xg supernatant was incubated with 0.34 nmol (^{3}H)8-azido-cAMP (10.2 Ci/mmol) in the absence or presence of 14,000 nmol of unlabelled cAMP. The mixture was then irradiated with an Osram HBO 100 w/w-2 high pressure mercury lamp. A 1 cm layer of saturated $CuSO_4$ solution was positioned between the lamp and the sample. The samples were kept on ice at a distance of 5 cm from the lamp during the irradiation (18 min). The incubation was terminated by the addition of 0.1 ml sample-buffer, the proteins were separated on SDS-PAGE (8-15% polyacrylamide gradient) and the gel was prepared for autoradiography.

CHAPTER 4 THE MECHANISM OF ACTION OF LH

4.1 Introduction

The pituitary hormone LH is the main regulator of Leydig cell steroidogenesis. It is generally accepted that LH exerts its effect through activation of adenylate cyclase resulting in elevated levels of cAMP, activation of cAMP-dependent protein kinase and phosphorylation of specific proteins (Cooke et al, 1976; Bakker et al, 1983b). However, the causal and obligatory interrelationship(s) between these parameters have not been completely elucidated. Several reports in the literature have described that the correlation between the effects of LH or hCG (which is thought to have the same mode of action as LH) on cAMP levels and steroid production is not always perfect. Dufau et al (1978) showed that in rat Leydig cells the ED_{50} of both cholera toxin and hCG for cAMP stimulation were approximately the same, whereas hCG was 60 times more potent than cholera toxin in stimulation of testosterone production. With isolated mouse Leydig cells it was found that isoproterenol stimulation resulted in a small elevation of cAMP levels without a concomitant stimulation of testosterone production, whereas a low dose of LH stimulated steroid production without any effect on cAMP (Cooke et al, 1982). These results indicate that the involvement of cAMP in LH regulation of steroid production is not completely clear. In this regard, we have studied the effect of inhibitors of adenylate cyclase on the stimulatory effect of LH on cAMP levels and steroid production in Leydig cells isolated from immature rats (appendix paper 1).

4.2 Effects of adenylate cyclase inhibitors

Isolated Leydig cells were incubated with two specific adenylate cyclase inhibitors: 9-(tetrahydro-2-furyl)adenine (TFA) (Haslam et al, 1978; Harris et al, 1979; Simchowitz et al, 1983) and 2'5'-dideoxyadenosine (DDA) (Fain et al, 1972;

Filetti & Rapoport, 1983; Florio & Ross, 1983). Both TFA and DDA inhibited LH-stimulated cAMP levels (appendix paper 1, figure 1 & 2). However, pregnenolone production was not inhibited, but rather increased in the presence of TFA as well as DDA. If cAMP is an obligatory mediator of the action of LH on steroid production, this increase in steroid production cannot be readily explained, because a decrease or no effect would have been anticipated. The observed results are similar to the synergistic effect of LHRH on LH-dependent steroid production (cf. chapter 5), although the mechanism of action of LHRH on cAMP levels on the one hand and of DDA and TFA on the other hand appear different: LHRH-A probably causes an attenuation of cAMP levels by activation of a phosphodiesterase (Sullivan & Cooke, 1984), whereas TFA and DDA act directly on the adenylate cyclase.

These results show that cAMP may not necessarily be the sole and obligatory second messenger in the action of LH on steroid production, albeit cAMP can be important in the mediation of the signal of LH to the cholesterol side chain cleavage. For instance, the phosphodiesterase inhibitor MIX potentiates the LH-dependent steroid production by immature rat Leydig cells (appendix paper 2, table 1), and dbcAMP can mimic many effects of LH (Bakker et al, 1983b). Since there may be more than one second messenger system involved in relaying the effects of LH on steroidogenesis, we have investigated the possible involvement of an alternative transducing system, i.e. the involvement of fluxes of calcium ions through the plasma membrane in the action of LH.

4.3 Involvement of calcium ions in the action of LH

The involvement of calcium was investigated by determining the effect on LH-stimulated steroid production of: 1) decreasing the extracellular calcium concentration, 2) blocking calcium channels with diltiazem, 3) inhibiting the activity of the calcium-binding protein calmodulin with trifluoperazine, and 4) artificially enhancing the intracellular free calcium concen-

tration using the calcium ionophore A23187. In all experiments the steroid production in the presence of 25-hydroxycholesterol was also measured, to obtain an indication of possible non-specific effects of the different treatments on the cholesterol side chain cleavage activity (Mason & Robidoux, 1978; Brinkmann et al, 1984).

In 3 h incubations, A23187 (0.1-10 μM) inhibited both LH- and 25-hydroxycholesterol-dependent pregnenolone production suggesting non-specific effects on the cholesterol side chain cleavage enzyme (appendix paper 2, figure 5). Treatment of the cells for 3 h with 0.1-1 μM A23187 progressively decreased ATP-levels in the cells, indicating that the ionophore had deleterious effects on immature rat Leydig cells (appendix paper 2).

A decrease of the extracellular calcium concentration resulted in a concomitant decrease of steroid production in the presence of LH (appendix paper 2, figure 3). The blocking of calcium fluxes with diltiazem (appendix paper 2, table 2), as well as inhibiting calmodulin action with trifluoperazine (figure 4.1) also partially inhibited LH action. These results

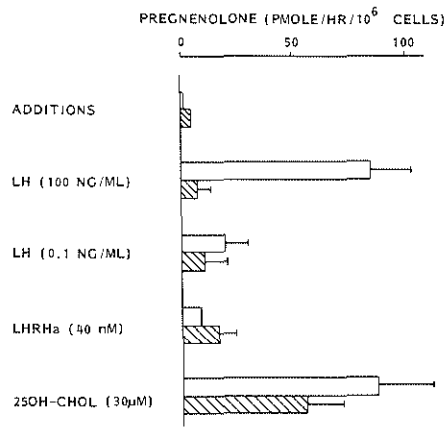

Figure 4.1 Effect of calmodulin-inhibitor trifluoperazine (TFP) on pregnenolone production by Leydig cells isolated from immature rats. Cells were incubated for 3 h with hormones as indicated, in the absence (open bars) or presence (cross-hatched bars) of 10 μM TFP.

show that calcium fluxes through the plasma membrane are involved in the effects of LH on steroid production. However, lowering the extracellular calcium concentrations to very low levels by the inclusion of the calcium chelator EGTA (0.1 mM), did not result in a complete block of LH stimulation, suggesting that the activation of the cholesterol side chain cleavage enzyme is not completely dependent on extracellular calcium. This suggestion is substantiated by the observation that EGTA could only inhibit 70% of the effects of both LHRH-A and a low concentration of LH. Addition of the different probes did not influence the 25-hydroxycholesterol-dependent steroid production, except in the case of trifluoperazine, suggesting that this compound has non-specific actions. It has been shown indeed, that trifluoperazine can have rather non-specific effects, since in platelets it inhibits PK-C directly (Sanchez et al, 1983).

The dependence of steroid production on the presence of extracellular calcium has been shown also in swine granulosa cells (Veldhuis et al, 1983), rat granulosa cells (Tsang & Carnegie, 1983), adrenocortical cells (Trzeciak & Mathé, 1981) and in mature rat Leydig cells (Janszen et al, 1976). Low extracellular calcium concentrations do not cause permanent cell damage, since the inhibitory effects of low calcium levels on Leydig cells (Janszen et al, 1976) and Sertoli cells (Oonk, personal communication) are reversible. The similarity of the effects of low extracellular calcium concentrations on the actions of LH and LHRH-A on steroid production suggests that extracellular calcium may influence the steroidogenic pathway at or distal to the point where the LH- and LHRH-A-dependent routes of activation meet.

An increase in the intracellular free calcium concentration can have several effects including for instance the activation of calcium-dependent protein kinases or changes in the cytoskeleton (see subsection 2.4). We have investigated the presence of calcium-activated protein kinases in a homogenate of Percoll-purified immature rat Leydig cells (figure 4.2). After incubation in the presence of (γ)-^{32}P-ATP with or without cal-

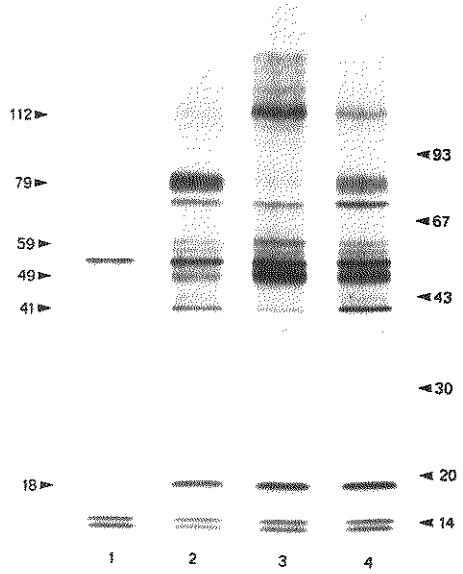

Figure 4.2 Pattern of in vitro protein phosphorylation. A homogenate of immature rat Leydig cells was incubated with $(\gamma)^{32}$P-ATP as described in section 3.4. Molecular weight markers are indicated on the right. Lane 1: control; lane 2: 2 mM $CaCl_2$; lane 3: 2 mM $CaCl_2$, 250 μg/ml partially purified calmodulin; lane 4: 2 mM $CaCl_2$, 250 μg/ml calmodulin, 50 μM TFP.

cium and calmodulin, the proteins were extracted and separated on SDS-PAGE. The autoradiograph of the dried gel showed calcium-dependent phosphorylations of proteins with relative molecular masses of 18, 41 and 79 kDa as well as calcium-calmodulin-dependent phosphoproteins of 49, 59 and 112 kDa. The effects of calmodulin on the phosphorylation could be inhibited specifically by trifluoperazine. Although the physiological significance of these proteins is not known, these results show the presence of two calcium-dependent kinase activities with endogenous substrates in Leydig cells.

4.4 Conclusions

The results presented in this chapter show that cAMP is not the sole and obligatory second messenger in the regulation of steroid production by LH. The influx of calcium ions may also be very important, and the results of the phosphorylation experiments with broken cell preparations show that the molecular mechanisms for the transduction of the calcium effects are present in the immature rat Leydig cell. The presence of extracellular calcium is necessary for a complete stimulation of steroid production. However, a small part of the action of LH and LHRH-A appears to be independent of extracellular calcium.

CHAPTER 5 MECHANISM OF ACTION OF LUTEINIZING HORMONE-RELEASING HORMONE

5.1 Introduction

The main physiologic function of the hypothalamic decapeptide LHRH is probably its effect on the release of LH from the pituitary, but it has been shown that LHRH can also act directly on gonadal steroid producing cells (for review: Hsueh & Jones, 1981). Not all steroidogenic cells are sensitive to LHRH. Rat Leydig cells give a good response (appendix paper 2), but gonadal cells of murine and human origin show no response at all, and no LHRH-receptors have been detected in the tissue from these species (Casper et al, 1982, 1984; Clayton & Huhtaniemi, 1982; Hunter et al, 1982). The physiological relevance of the direct action of LHRH on rat testicular cells remains unclear, since LHRH is not produced in significant amounts within the testis (Hedger et al, 1985). However, the mechanism of action of LHRH is interesting, since it has been found to be completely different from the effect of LH. LHRH has not only a direct effect on basal steroid production in isolated rat Leydig cells, but also affects the action of LH (Hunter et al, 1982; Rommerts et al, 1984b; appendix paper 2). The LHRH controlled pathways may therefore provide an additional system for the regulation of steroidogenesis. Although LHRH appears not to be the physiological modulator of Leydig cells in vivo, it may be that LHRH controlled pathways are activated under physiological conditions by other locally produced peptides. Hence, a study of the mechanism of action of LHRH on Leydig cell steroidogenesis may elucidate yet unknown regulatory pathways which might play a role in the physiological control of Leydig cell function.

The effects of LHRH on rat Leydig cells are mediated by specific high affinity receptors for LHRH on Leydig cell membranes (Sharpe & Fraser, 1980; Clayton et al, 1980). Studies with isolated Leydig cells show a biphasic effect of LHRH and its ago-

nists (LHRH-A) on steroid production. In short term incubations (less than 3 h) LHRH-A stimulates both basal and LH-dependent steroid production, but when cells are incubated for 24 h or longer with LHRH-A, the LH-dependent steroidogenesis is inhibited by the releasing hormone (Hunter et al, 1982; Browning et al, 1983; Rommerts et al, 1984b).

The mechanism of action of LHRH on Leydig cell steroid production is unclear. Cyclic nucleotides are apparently not involved in LHRH action on Leydig cells (Lin, 1984; appendix paper 2). In pituitary gonadotrophs, LHRH stimulates PIP_2 breakdown (Andrews & Conn, 1986) and a rise in inositol phosphates (Kiesel et al, 1986), and it has been implied that PK-C may be involved in LHRH action in these cells (Hirota et al, 1985). With an interstitial cell preparation isolated from testes of mature rats, Molcho et al (1984a) have shown that LHRH-A stimulates PI-turnover. Because regulation of PI-turnover has been shown to be involved in calcium-mediated actions (Downes & Michell, 1985; section 2.3), we have considered the possibility that the effect of LHRH on PI-turnover could reflect that calcium might be involved in the action of LHRH on steroid production. In this regard we have investigated the involvement of extracellular calcium and calcium fluxes through the plasma membrane in the action of LHRH on Leydig cell steroidogenesis (section 5.2 and appendix paper 2).

5.2 Involvement of calcium ions in the action of LHRH

To study the involvement of calcium ions in the action of LHRH-A, we have incubated the cells in medium with a low calcium concentration (appendix paper 2, figure 3). The low calcium concentration resulted in an inhibition of LHRH-A-stimulated steroid production which was comparable to the effect of this medium on LH-dependent pregnenolone production (cf. section 4.3). However, blocking of calcium channels with diltiazem (appendix paper 2, figure 4), and inhibition of calmodulin action with trifluoperazine (figure 4.1) had no effect on LHRH-A stimulation of steroid production, whereas these

treatments resulted in an inhibition of the effect of a submaximally stimulatory dose of LH. These results suggest that the presence of calcium may be essential for the transduction of any stimulatory signal to the CSCC, but that calcium fluxes may be involved only in the action of LH. The calmodulin inhibitor may have exerted non-specific effects on Leydig cells (see section 4.3). From the present results it cannot be excluded that calcium release from intracellular stores is essential for the action of LHRH. This release may be triggered by IP_3 released from PIP_2 in the plasma membrane after stimulation of the cells with LHRH-A. However, until now only changes in turnover of PI have been studied (Molcho et al, 1984a), and there is no evidence that IP_3 is produced in Leydig cells upon stimulation by LHRH. In this respect it may be of interest that the change of intracellular calcium levels in mature Leydig cells (measured with the Quin-II method) in response to LH or LHRH-A, showed a lag time of approximately 2 minutes after stimulation with LH, whereas no lag time was apparent upon addition of LHRH-A (Sullivan & Cooke, 1986). This suggests that LH may trigger the slower entry of calcium through a calcium channel, whereas LHRH-A, through IP_3 formation may trigger calcium release from intracellular stores, which is generally a much faster process (Reinhart et al, 1984; Downes & Michell, 1985). These considerations lead towards the suggestion that PIP_2-hydrolysis may be involved in LHRH action. Apart from calcium release from the endoplasmic reticulum, this pathway probably also employs activation of PK-C through the production of diacylglycerol (cf. section 2.3 and figure 2.3). We have investigated this possibility by comparing the effects of phorbol ester and phospholipase C, both known activators of PK-C, and of LHRH-A on steroid production, protein phosphorylation and protein synthesis (section 5.3 and appendix paper 4).

5.3 Effects of phorbol ester and phospholipase C

5.3.1 pregnenolone production

LHRH-A has a striking synergistic effect on LH-dependent pregnenolone production (appendix paper 2, figure 1). LH alone stimulates steroid production 35 fold, but this effect can be enhanced further (60 fold) by the inclusion of LHRH-A in the incubation medium, whereas LHRH-A alone stimulates pregnenolone production only 5 fold (appendix paper 2, figure 2). 4β-Phorbol-12-myristate-13-acetate (PMA) caused effects completely different from those of LHRH-A. PMA alone stimulated steroid production 3 fold, but inhibited the effect of intermediate concentrations of LH, without affecting the steroid production in maximally stimulated cells (appendix paper 4, figure 2). When PMA was added to Leydig cells in the presence of different concentrations of dbcAMP, the stimulatory effects of PMA and dbcAMP were additive, suggesting that the inhibitory effect of PMA on LH-stimulated steroid production occurred before the formation of cAMP, and also that PMA did not have deleterious effects on the cells (appendix paper 4, figure 2). These results are consonant with observations in mouse Leydig cells, where it has been shown that the site of inhibition by PMA of hCG effects on adenylate cyclase is localized at the regulatory GTP-binding protein of the adenylate cyclase system (N_s) (Mukhopadhyay & Schumacher, 1985).

We have also used phospholipase C (PL-C) to study the effects of phospholipid hydrolysis on Leydig cell steroid production. PL-C is thought to activate PK-C. There is no direct evidence for the action of PL-C on PK-C, but such an action has been suggested from the similarity of the effects of PMA and PL-C on protein phosphorylation in intact cells, and from the ability of PL-C to stimulate the levels of diacylglycerol in cell membranes (Rozengurt et al, 1983; Hadjian et al, 1984; Fischer et al, 1985; Jaken, 1985). We have used PL-C from Clostridium perfringens (type XII), because this enzyme has been shown by Fischer et al (1985) to be effective, rather than

the enzyme from Bacillus cereus which was not effective in murine epidermal cells (see also Chap et al, 1977; appendix paper 4).

PL-C stimulated steroid production 3 fold, and acted synergistically in stimulating LH-dependent pregnenolone production 2 fold (appendix paper 4, figure 1) . These results showed that the LHRH effect can be mimicked by PL-C, but not by PMA, suggesting that the mechanisms of action of PL-C and PMA are different in Leydig cells, although both are thought to activate the PK-C pathway. The difference between the effects of PL-C and PMA on pregnenolone production (and protein phosphorylation and protein synthesis, see below) might arise from the existence of different types of PK-C in the cell (Kiss & Luo, 1986; de Jonge, personal communication). These results and the following results obtained from experiments on protein phosphorylation and protein synthesis are summarized in Table 5.1. For the exact figures the reader is referred to appendix paper 4.

Table 5.1 Comparison of the effects of different stimuli on protein phosphorylation, protein synthesis and pregnenolone production in immature rat Leydig cells.

Activity	Activator			
	LH	LHRH-A	PMA	PL-C
Steroid production				
without added LH	-	6x	3x	3x
in the presence of LH	25x	50x	25x*	50x
Protein phosphorylation				
17 K	+++	0	+++	0
33 K	+++	0	++	0
Protein synthesis				
14 K	+++	+	++	+
27/30 K	+++	+	++	+

*: PMA inhibits pregnenolone production stimulated by submaximally stimulatory concentrations of LH.

5.3.2 protein phosphorylation

Bakker et al (1983b) have shown in Leydig tumour cells that LH-stimulated pregnenolone production is accompanied by the enhanced phosphorylation of at least six phosphoproteins with molecular weights ranging from 17 to 76 kDa. The most pronounced effect of LH was on the phosphorylation of a nuclear 17 kDa and the 33 kDa ribosomal protein S6. hCG-dependent phosphorylation of proteins in the same molecular weight range has also been shown in rat Leydig cell extracts (Dufau et al, 1981) and in intact porcine Leydig cells (Dazord et al, 1984). In intact immature rat Leydig cells, we also observed the LH-stimulated phosphorylation of the 17 and 33 kDa proteins (appendix paper 4, figure 3). The phosphorylation of these proteins was also stimulated by PMA to approximately the same extent as the phosphorylation obtained with LH. PL-C and LHRH-A did not have any effect on protein phosphorylation (appendix paper 4, figure 3; table 5.1). These results show that there is no comparable effect of PMA and PL-C on protein phosphorylation and

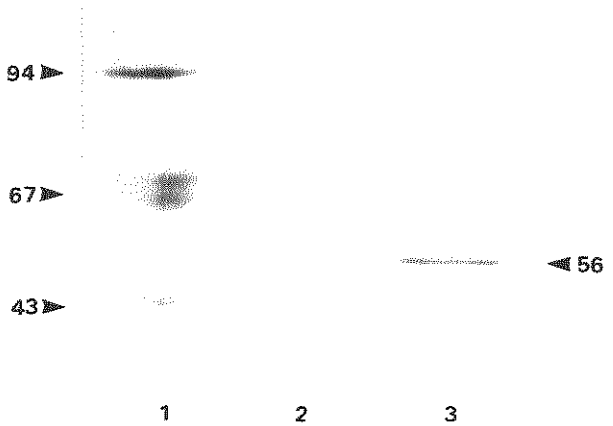

Figure 5.1 Fluorograph of proteins labelled with (^{3}H)8-azido-cAMP. A homogenate of Leydig tumour cells with (^{3}H)8-azido-cAMP was irradiated in the absence (lane 3) or the presence (lane 2) of an excess unlabelled cAMP. Lane 1: Marker proteins labelled with (^{14}C)-formaldehyde according to Jentoft & Dearborn (1979).

stimulation of steroid production.

The stimulation of phosphorylation of 17 and 33 kDa proteins by PMA suggests that these proteins are phosphorylated directly by PK-C. Bakker et al (1984) observed a discrepancy between the stimulation of phosphorylation of the 17 kDa protein by LH and dbcAMP, and the ability of these compounds to stimulate steroid production by tumour Leydig cells. The effect of dbcAMP on the phosphorylation of the 17 kDa protein was almost non-existent when compared to the effect of LH, whereas stimulation of steroid production by LH was less pronounced than the stimulation in the presence of dbcAMP (Bakker et al, 1984). These observations may be explained by activation of PK-C by LH, but not by dbcAMP, which results in the stimulation of the phosphorylation of the 17 kDa protein.

In the case of the ribosomal protein S6 the effect of PMA could also be a direct phosphorylation of S6 by PK-C. It has been shown that S6 can be phosphorylated by both PK-A and PK-C (Burkhard & Traugh, 1983; Trevillyan et al, 1984), and this has led to the suggestion that ribosomal protein S6 can be a site of multiple regulation of protein synthesis through phosphorylation at different, protein kinase specific sites (Traugh, 1981; Padel & Söling, 1985). There may still be another explanation for the effects of PMA on S6 phosphorylation. PMA also has a small effect on the phosphorylation of a 57 kDa protein, which may be the regulatory subunit (R_{II}) of PK-A_{II} (Cooke et al, 1979a). Phosphorylation of R_{II} may have a stimulatory effect on PK-A (see section 2.2.3). Therefore, we have investigated the identity of the 57 kDa protein and whether the phosphorylation of this 57 kDa protein could be stimulated by PMA in Leydig tumour cells. These studies might provide evidence for activation of PK-A by the PK-C pathway.

The identity of the 57 kDa protein was established in two independent ways: in a homogenate of Leydig tumour cells cAMP-binding proteins were labelled with the photo-affinity probe (^{3}H)8-azido-cAMP (figure 5.1), and a Western-type blotting experiment using a specific anti-R_{II} serum was performed (figure 5.2). Both experiments resulted in the specific labelling of a

single 57 kDa protein. These results show that the 57 kDa protein is R_{II}. Protein phosphorylation experiments in intact Leydig tumour cells also showed increased labelling of the 57 kDa protein in the presence of LH and PMA (figure 5.3). Preliminary results from investigations on protein phosphorylation in Leydig tumour cell homogenates in the presence of $(\gamma)^{32}$P-ATP showed a cAMP-dependent phosphorylation of a 57 kDa protein. Furthermore, under conditions favourable for activation of PK-C (subsection 3.4), we could show that phosphorylation of R_{II} occurred, whereas this phosphorylation was absent under control conditions (results not shown).

From these results we have concluded that stimulation of PK-C by PMA in intact cells may lead also to activation of PK-A via phosphorylation of R_{II}. In this way the phosphorylation of S6 might be catalyzed by PK-A, which in turn is activated by PK-C.

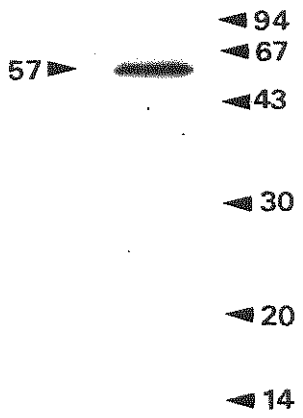

Figure 5.2 Autoradiograph of a Western blot, incubated with a specific anti R_{II} serum, The autoradiograph of the filter incubated with control serum did not show any radioactive bands (not shown). Molecular weight markers are indicated on the right.

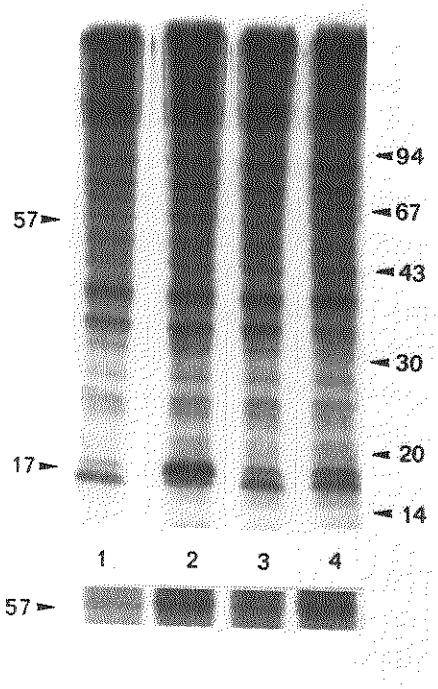

Figure 5.3 Protein phosphorylation pattern of intact tumour Leydig cells. Cells were incubated for 3 h in the presence of $^{32}PO_4$. Lane 1: control; lane 2: 100 ng/ml LH; lane 3: 40 nM LHRH-A; lane 4: 100 ng/ml PMA. Molecular weight markers are indicated on the right. Inset: detail of the same gel.

5.3.3 protein synthesis

Protein synthesis experiments which may reflect hormonal effects on Leydig cell in a different way, revealed a stimulation of the synthesis of proteins with molecular weights of 14, 27 and 30 kDa by LH, LHRH-A, PMA and PL-C (appendix paper, figure 3). Janszen et al (1978) observed in immature rat Leydig cells that LH stimulated the synthesis of 27 and 29 kDa proteins, which probably are the same proteins as the 27 and 30 kDa proteins described in appendix paper 4. These authors also showed the LH-stimulated synthesis of 11 and 21 kDa proteins, which were not apparent in our experiments. However, we investigated only the proteins in a 10,000xg supernatant of sonicated cells,

and the 11 and 21 kDa proteins may have been removed during the fractionation procedure. The effects of LH on the 14, 27 and 30 kDa proteins are much more pronounced than the effects of LHRH-A, PL-C and PMA, but we have not correlated the intensity of the newly synthesized protein bands with the potency of the agonist to stimulate steroidogenesis in a quantitative manner. In this respect, it is not possible to conclude whether protein synthesis patterns might be a better reflection of the ability of an agonist to stimulate steroid production than protein phosphorylation patterns. There is a difference in the kinetics of the stimulation of synthesis of specific proteins, which is apparent only after a 5 h incubation period, and the kinetics of the stimulation of steroid production, which takes anywhere from 2 min for the initial effect of LH (Rommerts et al, 1982b) to 3 h for LHRH-A to reach its maximal effect (section 3.2). Hence, although the activation of steroid production is dependent on protein synthesis in Leydig cells (Cooke et al, 1975), a direct correlation between steroidogenesis and the identity of newly synthesized proteins remains to be established. Other, as yet unidentified proteins, which might be synthesized more rapidly, might play a more important role in the regulation of steroidogenesis in Leydig cells (cf. Losier & Younglai, 1981; Pedersen & Brownie, 1983).

5.4 Conclusions

LHRH stimulation of rat Leydig cell steroid production is independent of LH action and does not involve cyclic nucleotides. Both LHRH-A and PL-C did stimulate LH-dependent pregnenolone production synergistically, and the effects of these compounds on protein phosphorylation and protein synthesis were similar. These results suggest that the primary effect of LHRH is probably on phospholipid hydrolysis, possibly resulting in the production of IP_3.

CHAPTER 6 GTP-BINDING PROTEINS IN MATURE RAT LEYDIG CELLS

6.1 Introduction

It was discussed in chapter 2 that GTP-binding proteins (N proteins) are involved in the transduction of the signal from several hormone-receptor complexes to the intracellular effector system. One of the actions of LH in Leydig cells is the stimulation of the production of cAMP, and, similar to other adenylate cyclase-stimulating hormones, the effect of LH appears to be mediated by the stimulatory N protein (N_s). This can be inferred from the stimulating effects of cholera toxin, which specifically activates N_s, on cAMP production and steroidogenesis in intact Leydig cells (Cooke et al, 1977; Dufau et al, 1978). Furthermore, the observation that hCG-stimulation of adenylate cyclase in Leydig cell membrane preparations is dependent on the presence of GTP (Dufau et al, 1980; Mukhopadhyay & Schumacher, 1985), is indicative that the effect of LH (hCG) on adenylate cyclase is mediated by a GTP-binding protein, possibly N_s. The inhibiting GTP-binding protein, N_i, may also be involved in the action of adenylate cyclase. In reconstitution experiments with N_s, N_i, adenylate cyclase and β-adrenergic receptor in liposomes Cerione et al (1985) showed that the activated receptor may have a dual role, i.e. 1) activation of N_s, and 2) removal of the inhibition of adenylate cyclase by N_i. Either one or both of these actions may result in a significant increase in the hormonal effect on cAMP production.

In this section both the presence and functional properties of N_s and N_i in Leydig cells isolated from mature rats have been investigated, in order to establish whether Leydig cells may be controlled by these different GTP-binding proteins.

6.2 Presence of N_s and N_i in Leydig cells

To study the presence of N_s and N_i in rat Leydig cells, we have used a membrane preparation obtained from Leydig cells isolated from mature rats. This cell preparation was used because sufficient amounts of Leydig cells of high purity could only be obtained from mature rats. A crude membrane preparation was incubated with either cholera toxin or pertussis toxin in the presence of (^{32}P)-NAD. Cholera toxin stimulates ADP-ribosylation of the α_s-subunit of N_s in the presence of Mg^{2+} and GTP, and there are indications that cholera toxin has a preference for the activated form of N_s (Gill, 1977). The α-subunit of N_i is ADP-ribosylated by pertussis toxin. This toxin has a preference for the form of N_i that predominates in the absence of Mg^{2+}, and the ADP-ribosylation can be stimulated by GDP (Ribeiro-Neto et al, 1985).

Incubation of the membrane preparation with pertussis toxin in the presence of (^{32}P)-NAD and GDP resulted in a pertussis toxin-dependent labelling of a 39 kDa protein (appendix chapter, figure 1). In the presence of GTPγS and $MgCl_2$ instead of GDP the ADP-ribosylation of this protein was decreased (not shown). Similar experiments with cholera toxin showed labelling of a 44 kDa protein in the presence of GTP (appendix chapter, figure 1). Western blotting experiments, using a polyclonal antibody against the β-subunit, revealed that the β-subunit which is identical in both N_s and N_i was also present (results not shown).

6.3 Effects of cholera toxin and pertussis toxin on Leydig cell steroid production

We have also investigated the functional importance of N_s and N_i in Leydig cells isolated from 6-8 week old rats. Isolated Leydig cells were preincubated for 2 h in the presence of either cholera or pertussis toxin (100 ng/ml). Preincubation with the toxins for this period of time is necessary for the activation or inhibition of N_s or N_i, respectively. After the

preincubation period LH (100 ng/ml) or LHRH-A (40 nM) were added, and the pregnenolone production during the following 3 h was determined (appendix chapter, figure 2). Cholera toxin stimulated both basal and LHRH-A-dependent pregnenolone production, but did not affect steroid production in the presence of LH. Preincubation of Leydig cells with pertussis toxin for 2 h did not have any effect on steroid production. In contrast, treating the cells for 24 h with pertussis toxin resulted in a stimulation of basal, LH- and cholera toxin-dependent pregnenolone production (appendix chapter, figure 2). LHRH-A did not have stimulatory effects on steroid production after the cells had been incubated for 24 h (Hunter et al, 1982; Rommerts et al, 1984b).

6.4 Discussion

The results show that functionally active N_s and N_i are present in Leydig cells from mature rats. Cholera toxin stimulated the ADP-ribosylation of a 44 kDa protein in a crude Leydig cell membrane preparation. The dependency of the labelling on cholera toxin, and the observed molecular weight strongly suggest that this protein is α_s (Kaslow et al, 1980; Gilman, 1984). Pertussis toxin stimulated the ADP-ribosylation of a 39 kDa protein, and this labelling was decreased in the presence of $MgCl_2$ and GTPγS, suggesting that this protein is the α-subunit of N_i (Bokoch et al, 1983; Murayama et al, 1983; Ribeiro-Neto et al, 1985). In addition, we have observed that the α-subunit of N_s and N_i is present in the Leydig cell membrane preparations.

The stimulation of steroid production in Leydig cells by LH is accompanied by increased levels of cAMP and activation of PK-A (Cooke et al, 1976; Podesta et al, 1978). Cholera toxin stimulates cAMP levels in Leydig cells (Cooke et al, 1977; Dufau et al, 1978) probably through activation of N_s. This is substantiated by the observation that cholera toxin treatment of Leydig cells resulted in stimulation of LHRH-A-dependent pregnenolone production. N_s, activated by cholera toxin, may

have stimulated adenylate cyclase. The increase in cAMP levels resulted in a stimulation of LHRH-A-dependent steroid production, since the action of LHRH-A on Leydig cells is independent of cAMP (Sullivan & Cooke, 1984; appendix paper 2).

N_i may have a multiple role. In reconstitution experiments it has been shown that N_i inhibits the basal adenylate cyclase activity much more than the stimulated enzyme activity (Cerione et al, 1985). Its presence in the plasma membrane may be necessary to assure a maximal stimulatory effect of hormones on adenylate cyclase. After a 2 h preincubation period we were not able to show effects of pertussis toxin on steroid production. However, when pertussis toxin was present for 24 h, the pregnenolone production in control cells and in the presence of LH or cholera toxin was stimulated. These results show that N_i is involved in the regulation of steroid production in rat Leydig cells. It is not clear whether the effect of pertussis toxin evolves slower compared to the effect of cholera toxin. It has been observed in other cell systems that the effect of pertussis toxin may take from 60 min to 24 h to become maximal (Adashi et al, 1984; Holz IV et al, 1986). Alternatively, the Leydig cells may have acquired a sensitivity to pertussis toxin during culture. In this respect it is of interest that Leydig cells in culture become sensitive to adenosine (Rommerts et al, 1984a) and β-adrenergic agonists (Anakwe et al, 1985). N_i might also mediate the coupling of receptors of the R_i-type (cf. section 2.2) to inhibition of adenylate cyclase. It has been shown that 24 h treatment of cultured testicular cells with pertussis toxin abolished the inhibitory effects of arginine-vasotocin on hCG-dependent steroid production, suggesting that the receptor for arginine-vasotocin may be of the R_i-type (Adashi et al, 1984). A third role of N_i might be the coupling of receptors to hormone-regulated calcium-channels (Okajima et al, 1985; Koch et al, 1985; Schultz & Rosenthal, 1985; Holz IV et al, 1986), but it is not clear whether N_i functions in this manner in Leydig cells. There is no evidence, that N_i is involved in the action of LHRH on Leydig cell steroid production, because there are only indications

that the effects of LHRH are mediated by an increase in PI-turnover (Molcho et al, 1984a; cf. section 5.3), and this pathway is not activated by N_i (Downes & Michell, 1985).

6.5 Conclusions

Two GTP-binding proteins, N_s and N_i, are present in mature rat Leydig cells, and both N proteins are involved in the regulation of steroid production. N_s may be required for coupling of the LH-receptor to adenylate cyclase. N_s and N_i appear to play no role in the action of LHRH on pregnenolone production.

CHAPTER 7 GENERAL DISCUSSION

7.1 Introduction

In the preceding chapters the results of our investigations concerning different second messenger systems operating in testis Leydig cells have been presented and discussed. In this chapter a model for the mechanism of the regulation of steroid production consistent with our results will be presented. Figure 7.1 gives a schematic representation of this model. The two main pathways triggered by LH and LHRH are described in sections 7.2.1 and 7.2.2 respectively. The different ways by which these hormones may stimulate cholesterol side chain cleavage will be discussed in section 7.2.3. The chapter concludes with some comments about the specific end-point parameter of Leydig cell function and the relative importance ascribed to regulators of the Leydig cell.

7.2 A model for the regulation of steroid production

7.2.1 mechanism of action of LH

The binding of LH to its receptor results in the activation of two main pathways, viz. the stimulation of adenylate cyclase and an increase in intracellular calcium levels. These distinct effects of LH might be mediated by either two different receptor molecules, each coupled to its own N protein, or, alternatively, the receptor molecule might interact with two different N proteins. Until now there is no evidence based on binding studies that there are different receptor molecules for LH (Dufau et al, 1973). In addition, studies on different receptor molecules may be complicated by metabolism of the receptor after internalization in intact cells (Habberfield et al, 1986), and proteolytic modification when membranes are used (Kellokumpu & Rajaniemi, 1985; Ascoli & Segaloff, 1986). The stimulation of adenylate cyclase by LH may be mediated by N_s.

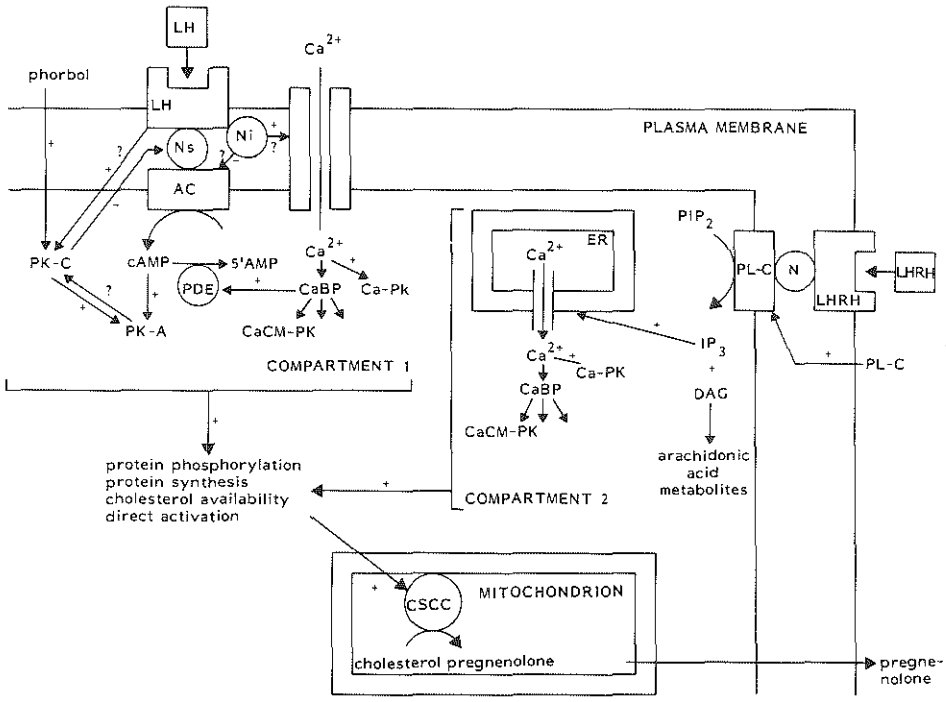

Figure 7.1 A model for the regulation of steroid production in the Leydig cell by LH and LHRH. For a complete explanation see text.
In summary: LH binds to its receptor resulting in activation of N_s and N_i. N_s stimulates the activity of adenylate cyclase, and cAMP stimulates PK-A. N_i may inhibit adenylate cyclase, but might also stimulate a calcium-channel, resulting in an increase in intracellular calcium levels. Calcium may activate a calcium- or a calcium-calmodulin-dependent protein kinase. Calcium could also activate phosphodiesterase resulting in a decrease of cAMP levels. LH might also stimulate the activity of PK-C, which in turn could activate PK-A. LHRH binds to its receptor resulting in activation of the PI-system. IP_3 may release calcium ions from intracellular stores. The calcium may activate protein kinases, or may stimulate other functions in the cell via calcium-binding proteins. The role of PK-C or arachidonic acid metabolites is not clear. Stimulation of cholesterol side chain cleaving activity may occur via these regulatory systems through stimulation of protein phosphorylation, protein synthesis, cholesterol availability or direct activation of the enzyme.
Abbreviations: N_s: stimulatory guanine nucleotide binding protein; N_i: inhibitory guanine nucleotide binding protein; AC: adenylate cyclase; PIP_2: phosphatidyl inositolbisphosphate; IP_3: inositol trisphosphate; DAG: diacylglycerol; PDE: phosphodiesterase; CaBP: calcium-binding protein; ER: endoplasmic reticulum CaCM-PK: calcium-calmodulin-dependent protein kinase; Ca-PK: calcium-dependent protein kinase; CSCC: cholesterol side chain cleaving enzyme; PL-C: phospholipase C; PK-C: protein kinase C; PK-A: cAMP-dependent protein kinase.

We have shown that the α_s-subunit of N_s is present in Leydig cells (chapter 6), and it has been shown by others that LH-stimulation of adenylate cyclase in Leydig cell membranes is dependent on the presence of GTP (Dufau et al 1980; Mukhopadhyay & Schumacher, 1985). Furthermore, cholera toxin which specifically activates N_s, stimulates cAMP levels (Cooke et al, 1977; Dufau et al, 1978) and pregnenolone production in Leydig cells (chapter 6). LH also stimulates intracellular free calcium levels (Sullivan & Cooke, 1986), and we have shown that calcium channels may be involved in this effect of LH (chapter 4). It has been suggested that N_i may be involved in the stimulation of hormone-regulated calcium channels, albeit this evidence is not direct, but is based on the inhibition of N_i by pertussis toxin (Okajima et al, 1985; Koch et al, 1985; Schultz & Rosenthal, 1985; Holz IV et al, 1986). The α-subunit of N_i is present in Leydig cells (chapter 6), and may have a similar function in the regulation of calcium channels as has been suggested for other cell systems. Another role of N_i could be the inhibition of adenylate cyclase (Birnbaumer et al, 1985), thus providing a mechanism for modulation of the adenylate cyclase system. We have shown a stimulatory effect of pertussis toxin on steroid production (chapter 6), suggesting that inhibition of N_i may lead to an increase in cAMP levels in Leydig cells. These results are consonant with the observations of Adashi et al (1984) who showed that pretreatment of testicular cells with pertussis toxin resulted in an enhancement of the effects of hCG on testosterone production. The presence of N_i may be necessary for establishing a low basal activity of adenylate cyclase in the cells, assuring a low steroid production by non-stimulated cells. In analogy with the experimental findings of Cerione et al (1985) observed with the β-adrenergic system, it may be that the LH-receptor complex evokes activation of N_s and inactivation of N_i, giving rise to a large effect of the hormone on cAMP levels.

The suggested dual role of the LH receptor (coupling to N_s and N_i) may provide an explanation for the numerous observations of discrepancies between the stimulatory effects of LH on

cAMP levels and steroid production (Moyle et al, 1975; Dufau et al, 1978; Cooke et al, 1977, 1982; appendix paper 1). The N_s or N_i pathway may be preferentially stimulated depending on the properties of the phospholipids and proteins in the membrane of the cell. In this way the receptor can be in different "coupling states", providing the cell with different modes of regulation. In this respect, the results of Anakwe et al (1985) are of special relevance. They showed that in freshly isolated Leydig cells β-adrenergic receptors are present, but these receptors could not stimulate cAMP levels or steroid production. However, when the cells had been incubated for 3 h or longer, the coupling between receptors, cAMP and testosterone production could be demonstrated. These results show that mechanisms operate in the cell which can alter the coupling of receptors to their second messenger systems. The difference in effects of hCG and the hormone after partial removal of its sugar residues on either cAMP levels or steroid production in Leydig cells, suggests that the carbohydrate moiety of hCG and perhaps also of LH may be involved in the specificity of the coupling of the receptor to the intracellular messenger system (Moyle et al, 1975).

The increase in cAMP levels after stimulation with LH causes the activation of PK-A, and this kinase can phosphorylate many proteins in the cell, which in turn may stimulate protein synthesis or alter the cytoskeleton. The consequence of these events for the stimulation of steroid production is unknown at present, although it has been shown that continuous protein synthesis is required for the stimulation of steroid production (cf. subsection 7.2.3). Another effect of cAMP may be the stimulation of the calcium channel as was shown in heart muscle cells with 8-bromo-cyclic AMP (Cachelin et al, 1983) and in Leydig cells using dbcAMP (Sullivan & Cooke, 1986). However, in view of observations that LH can have effects without any elevation of cAMP (see references given above), it is not likely that increases in cAMP offer the only mechanism of stimulation of calcium influx by LH.

The increase in the intracellular free calcium concentration

can result in activation of calmodulin-dependent phosphodiesterases which can inhibit or turn off the cAMP signal (Erneux et al, 1985). Furthermore, we have shown that both calcium-dependent and calcium-calmodulin-dependent protein kinases which may be stimulated by increased levels of calcium and cause protein phosphorylation even in the absence of cAMP are present in Leydig cells. On the other hand, calcium-regulated protein kinases may also act together with cAMP-regulated protein kinases. In this respect, it has been shown that myosin light-chain kinase, which is dependent on Ca^{2+}-calmodulin, is a substrate for PK-A reducing its affinity for calmodulin (Lohmann & Walter, 1984; Manalan & Klee, 1984). Similarly, calcium and cAMP may also have effects on protein synthesis, since it has been shown that the phosphorylation of ribosomal protein S6, which may play a role in the regulation of protein synthesis (Traugh, 1981), can be stimulated by both cAMP- and calcium-dependent agonists (Padel et al, 1983).

7.2.2 mechanism of action of LHRH

The second receptor depicted in figure 7.1, the LHRH-receptor, may be coupled to the PI-turnover signalling system. This suggestion is based mainly on the similarity between the effects of LHRH-A and PL-C on Leydig cells and the absence of effects of calcium channel-blockers on LHRH-A stimulation of steroid production in Leydig cells (chapter 5). This may indicate that LHRH-A stimulates intracellular calcium concentrations not via influx through the plasma membrane, but by mobilization of calcium from intracellular stores. The absence of a lag-time in the rise in intracellular calcium after addition of LHRH-A to Leydig cells (Sullivan & Cooke, 1986) as well as the stimulation of PI-turnover by LHRH-A in an interstitial cell preparation (Molcho et al, 1984a) further substantiates this model. In analogy with other systems it may be possible that upon binding of LHRH to the receptor the cleavage of PIP_2 by a phospholipase C is triggered through activation of a putative N protein (Downes & Michell, 1985). The products of this

metabolic conversion, IP_3 and DAG, might stimulate calcium release from the endoplasmic reticulum and activate PK-C, respectively. We have no indications, however, that LHRH actually stimulates PK-C through this pathway. On the other hand phorbol esters do stimulate protein phosphorylation in Leydig cells, a finding which supports the presence of PK-C (chapter 5; see also Hirota et al, 1985). The similarities between the phosphorylation patterns obtained with LH and PMA (chapter 5), lead to the suggestion that LH instead of LHRH might stimulate PK-C activity in addition to intracellular cAMP and calcium. In this respect, it has been reported that LH can also stimulate the turnover of various phospholipids in the Leydig cell (Lowitt et al, 1982), although the effects of LH were small, and these observations have not been confirmed by other investigators. It remains to be elucidated whether and how PK-C is activated by LH in Leydig cells. PK-C may activate PK-A by phosphorylation of the R_{II} subunit of PK-A (chapter 5), and may inhibit stimulation of adenylate cyclase at the level of N_s (Mukhopadhyay & Schumacher, 1985). In this way PK-C may interact with the cAMP-system at different levels.

It has been observed that LH and LHRH both cause an increase of intracellular free calcium levels (Sullivan & Cooke, 1986; chapter 4 and 5). However, LH and LHRH act independently on steroid production (chapter 5). Hence, if changes in calcium concentrations are involved in the stimulation of steroid production the effects of LH and LHRH on intracellular calcium levels occur via separate intracellular mechanisms. A physical separation may be possible. For example, LH may stimulate a calcium influx through the plasma membrane, whereas LHRH may stimulate calcium efflux from the endoplasmic reticulum via IP_3. It may well be that the target proteins of Ca^{2+} are located at or near the source of the calcium ions. In this way a functional compartimentalization of the effects of calcium released from different sources can be envisaged.

A third messenger involved in the possible actions of the PI-turnover system could be the arachidonic acid metabolites. It is not clear, however, whether these metabolites are an

endproduct of stimulation by LH and LHRH (Molcho et al, 1984a, b), or whether they are involved in the regulation of steroidogenesis. Studies with specific inhibitors of lipoxygenase and cyclo-oxygenase have revealed that products of the lipoxygenase pathway, but not the cyclo-oxygenase pathway, may be involved in the regulation of steroid production by LH and LHRH in mature rat Leydig cells (Sullivan & Cooke, 1985a). However, one of the arachidonic metabolites, leukotriene B_4, does not appear to be involved in the regulation of steroidogenesis by LH and LHRH (Sullivan & Cooke, 1985b). Moreover, incubation of Leydig cells with phospholipase A_2 did not have an effect on steroid production (chapter 5).

7.2.3 stimulation of cholesterol side chain cleavage

The mechanism of the regulation of cholesterol side chain cleavage (CSCC) by the intracellular messengers is not clear. CSCC is thought to be the key enzyme in steroidogenesis because the activity of this enzyme determines the rate of production of androgens in the Leydig cell (Simpson, 1979; Rommerts et al, 1984a). The phosphorylation of proteins catalyzed by different protein kinases may be important in the regulation of CSCC (Bakker et al, 1981, 1983a,b). However, the kinetics of the phosphorylation of the 17 kDa nuclear protein and the 33 kDa ribosomal protein S6 did not correlate very well with the kinetics of the stimulation of steroid production (Bakker et al, 1981), and the role of these and other phosphoproteins (20, 43 and 76 kDa), that may be involved in the cytoskeleton is not clear (Bakker et al, 1983a,b). We have shown that protein phosphorylation patterns after stimulation with LH and phorbol ester were very similar, whereas there was a 30-fold difference in the ability of LH and phorbol ester to stimulate pregnenolone production (chapter 5). A direct regulation of CSCC through phosphorylation of the enzyme by a intramitochondrial protein kinase (PK-C) has been suggested by Vilgrain et al (1984), although these authors did not show a direct effect of phosphorylation on the activity of CSCC. As yet, the me-

chanism of the effect of stimulation of protein phosphorylation on the activity of CSCC is not clear.

Protein synthesis appears to be obligatory in the stimulation of steroid production. The effects of ACTH and LH on steroid production are inhibited by inhibitors of protein synthesis (Schulster et al, 1974; Cooke et al, 1975; Rommerts et al, 1983). However, in our studies on protein synthesis we have not been able to show proteins that were synthesized fast enough to play an important role in the acute regulation of steroid production (chapter 5). In this respect the studies of Pedersen & Brownie (1983) are of interest. They have shown the synthesis of a small peptide in adrenocortical cells that, on the basis of kinetic studies might be involved in the rapid effect of ACTH on steroidogenesis. Moreover, recent studies with adrenal and luteal cells have revealed a 28 kDa protein that may play a role in the effect of ACTH or hCG on steroid production (Pon & Orme-Johnson, 1986). Other proteins less dependent on de novo synthesis may also be required for the full activity of CSCC. In this respect, it has been suggested that the 14 kDa sterol carrier protein (SCP_2) may be involved in the regulation of the availability of cholesterol to CSCC. Although the protein does not appear to act as a carrier protein, it is specifically located in steroid producing cells in the testis (van Noort et al, 1986), and it has stimulatory effects on pregnenolone production in isolated Leydig cell mitochondria (van Noort, unpublished observations). These observations suggest that SCP_2 may play an important role in the regulation of steroid production in Leydig cells.

7.3 Concluding remarks

In this thesis we have presented the reults of investigations on the possible transducing systems that could play a role in regulation of Leydig cell function by LH and LHRH action. In most cases pregnenolone production has been used as a parameter for testicular steroid production. This resulted in the observations that LHRH-A had small effects on basal activi-

ties, but stimulated synergistically the LH-stimulated steroid production. Phorbol esters, however, cause small effects on steroid production under both conditions. These results may suggest that the PK-C system, stimulated with phorbol esters may not have an important role in the regulation of functional properties of Leydig cells. However, the stimulation of production of androgens appears not to be the only effect of hormones on Leydig cells. Hormones may stimulate growth of Leydig cells, and Leydig cells can also be the source of products other than steroids, e.g. different products of the pro-opiomelanocortin system (Tsong et al, 1982; Margioris et al, 1983), prostaglandins (Molcho et al, 1984a,b) and products that affect testicular vascular permeability (Setchell & Rommerts, 1986; Veijola & Rajaniemi, 1986). These products may have important functions in the regulation of testis function, such as regulation of the permeability of blood-vessels or communication between Leydig cells and Sertoli cells. It might be possible that the PK-C pathway, as probed with phorbol ester, is involved more in the regulation of one of these activities than in the regulation of steroid production and that the stimulation of steroid production with phorbol esters represents an additional effect. Therefore, the studies described in this thesis should be interpreted only with respect to the regulation of steroidogenesis, and may not give a complete picture of the importance of these messenger systems for the regulation of all Leydig cell functions. To obtain a complete description of the regulation of Leydig cell functions, it is necessary to study other functional properties in addition to steroid production. Moreover, the development of specific probes for the different transducing systems, such as specific inhibitors of the PIP_2-phospholipase C and PK-C should be attempted.

Our results also show that new studies have to be designed to investigate the connection between the transmembrane signalling systems and the long term biological effect. For instance, it has been shown in long term incubations that hCG stimulates the synthesis of the different components of the CSCC complex (Anderson & Mendelson, 1985). Investigations have

been initiated to study the role of nucleic acids and protein synthesis (Cooke et al, 1979b; Rommerts et al, 1983), but at present no information is available on the mechanism through which the second messengers can stimulate the transcription of the CSCC genes. Investigations on this part of the hormonal signal transduction system may result in a better understanding of the complex, interdependent, multiple regulatory systems that are present in cells such as the Leydig cell.

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SUMMARY

The work described in this thesis has been concerned mainly with the elucidation of some of the different second messenger systems present in testicular Leydig cells, and the regulation of steroidogenesis through these systems. Leydig cells which are located in the interstitium of the testis are the source of androgenic steroids. The androgens are essential for the development of germinal cells and for the development of male sex characteristics. The production of steroids (steroidogenesis) in the Leydig cell depends on the activity of several enzymes. The rate determining step in steroidogenesis is the conversion of cholesterol into pregnenolone catalyzed by the cholesterol side chain cleavage enzyme complex (CSCC). The production of androgens is mainly under the control of the pituitary hormone luteinizing hormone (LH), which regulates the production of steroids through the activity of CSCC. Recently it has been demonstrated that, in addition to LH, other hormones and locally produced factors can also regulate steroid production in Leydig cells (chapter 1).

Protein hormones exert their effects on cells via an interaction with receptor proteins in the plasma membrane. The receptor activates other proteins in the cell membrane eventually resulting in the production of second messengers, which can activate protein kinases or other enzymes in the cell, and this may result in stimulation of activities such as protein phosphorylation, protein synthesis, alterations of the properties of the cytoskeleton. Stimulation of steroidogenesis in Leydig cells by LH is accompanied by an increase in adenylate cyclase activity, elevated levels of cAMP and increased phosphorylation of proteins. However, cAMP may not be the only mediator for the regulation of steroid production, because other effectors, such as luteinizing hormone-releasing hormone (LHRH), which can also stimulate steroid production, appear to utilize other second messenger systems. In chapter 2 some background information is given on those regulatory mechanisms that employ cAMP, phosphoinositide metabolites and calcium ions as second

messengers.

In chapter 4 experiments are described that show that inhibition of adenylate cyclase with adenosine derivatives did not result in an attenuation of the stimulation of steroidogenesis by LH, but rather potentiated LH effects. It is concluded that cAMP is not the sole second messenger involved in the regulation of steroid production by LH. The involvement of calcium ions was investigated by determining the effect of either decreasing the extracellular calcium concentration or blocking of calcium channels with diltiazem on LH-stimulated steroid production. It was found that stimulation of steroid production is partly dependent on the presence of extracellular calcium, and that calcium fluxes through the plasma membrane may be necessary for the mediation of the effect of LH.

In chapter 5 experiments on the mechanism of action of LHRH are described. cAMP is probably not involved in the actions of LHRH on steroid production, which appear to be independent of those of LH. The presence of extracellular calcium is necessary for the stimulatory effect of LHRH. However, LHRH may stimulate increases in intracellular free calcium through mobilization of calcium ions from intracellular stores, rather than by an increase in calcium flux through the plasma membrane. Experiments on the effects of LHRH, phorbol ester and phospholipase C on pregnenolone production, protein phosphorylation and protein synthesis revealed that the effects of LHRH and phospholipase C on these different activities were similar, whereas phorbol ester stimulated protein phosphorylation and protein synthesis approximately to the same extent as LH. The results indicate that the primary effect of LHRH may be on phospholipid hydrolysis, possibly resulting in the production of phosphoinositide metabolites, which may affect steroid production. It remains to be elucidated whether LH activates protein kinase C.

In chapter 6 investigations on the presence and functional properties of the stimulatory (N_s) and the inhibitory (N_i) guanine nucleotide binding proteins in Leydig cells are described. N_s and N_i may be involved in the dual regulation of adenylate

cyclase in many cell types. ADP-ribosylation experiments with cholera toxin or pertussis toxin revealed that both α-subunits of N_s and N_i are present in Leydig cells. Preincubation of Leydig cells with either cholera toxin or pertussis toxin for 24 h resulted in a stimulation of both basal and LH-stimulated steroid production. It is concluded that N_s may mediate the effect of LH on adenylate cyclase. N_i may also be involved in the regulation of steroid production, but its exact role remains to be elucidated.

In chapter 7 a model for the regulation of steroid production in Leydig cells is presented and discussed. LH binds to its receptor resulting in activation of N_s which stimulates adenylate cyclase activity. The product of adenylate cyclase, cAMP, stimulates cAMP-dependent protein kinase. LH may also stimulate calcium influx through the plasma membrane, possibly via activation of N_i. LHRH may stimulate phosphoinositide metabolism, and the product inositol trisphosphate releases calcium ions from intracellular stores. The role of the other phosphoinositide products, diacylglycerol and arachidonic acid metabolites is not clear. An intracellular compartimentalization of the effects of calcium is suggested, because both LH and LHRH increase intracellular calcium concentrations and the hormones act independently on steroid production. Calcium may activate protein kinases or may regulate other functions in the cell via calcium-binding proteins. The combined actions of the second messengers (cAMP, phosphatidylinositol metabolites and calcium) result in activation of CSCC through stimulation of either protein phosphorylation and/or protein synthesis, which may cause an increase in cholesterol availability or direct activation of the enzyme.

SAMENVATTING

Het onderzoek dat in dit proefschrift is beschreven, was gericht op de opheldering van de verschillende boodschappersystemen die in Leydig cellen aanwezig zijn, en de regulatie van de vorming van steroid hormoon productie via deze systemen. Leydig cellen, die zich in het interstitiele weefsel van de testikel bevinden, zijn de bron van steroid hormonen (androgenen) die van essentieel belang zijn voor de groei van de mannelijke geslachtscellen en voor de ontwikkeling van de mannelijke geslachtskenmerken. De vorming van steroid hormonen door de Leydig cellen is afhankelijk van de activiteit van verschillende enzymen, waarbij de snelheid van de productie wordt bepaald door de snelheid van de omzetting van cholesterol in pregnenolon door het cholesterol-zijketen splitsend enzym. De regulatie van de productie van androgenen door Leydig cellen geschiedt voor een belangrijk deel door luteiniserend hormoon (LH), dat uit de hypofyse afkomstig is. LH reguleert de productie van androgenen via de activiteit van het cholesterol-zijketen splitsend enzym. Steroidogenese door Leydig cellen wordt echter niet uitsluitend gereguleerd door LH; ook andere verbindingen die de Leydig cellen beinvloeden, hebben een effect op de steroid productie (hoofdstuk 1).

Veel hormonen oefenen hun werking op cellen uit via binding aan een specifiek eiwit (receptor) in de plasmamembraan. Deze receptor activeert dan andere eiwitten in de membraan, wat uiteindelijk resulteert in de productie van "boodschappers" met een laag molecuulgewicht. Deze tweede boodschappers kunnen eiwitkinases of andere enzymen in de cel activeren, hetgeen kan leiden tot een stimulering van verschillende activiteiten in de cel zoals eiwitfosforylering, eiwitsynthese of veranderingen in het cytoskelet. De stimulering van steroid productie in Leydig cellen door LH gaat gepaard met een verhoging van de activiteit van adenylaat cyclase, verhoogde niveaus van cyclisch AMP en een verhoogde activiteit van eiwitfosforylering. Het cyclisch AMP systeem is echter waarschijnlijk niet het enige boodschappersysteem dat betrokken is bij de regulatie van steroid productie.

Andere factoren die ook de steroid productie kunnen stimuleren, zoals LH afgifte bevorderend hormoon (LHRH), lijken via andere boodschapper systemen te werken. Hoofdstuk 2 geeft een samenvatting van de belangrijkste informatie over dié regulatiemechanismen waarbij cyclisch AMP, metabolieten van fosfatidylinositol en calcium ionen als tweede boodschappers betrokken zijn.

In hoofdstuk 4 zijn experimenten beschreven waaruit blijkt dat remming van adenylaat cyclase door adenosine derivaten niet resulteerde in remming van de effecten van LH, maar veeleer in een verhoging van de stimulering van steroid productie door LH. De vraag in hoeverre calcium ionen betrokken zijn bij de regulatie van steroid productie werd onderzocht door de effecten van verlaging van de extracellulaire calcium concentratie of het blokkeren van "kanalen" voor het transport van calcium door de plasmamembraan op de LH-gestimuleerde steroid productie te bepalen. Er werd gevonden dat stimulering van steroid productie gedeeltelijk afhankelijk is van de aanwezigheid van extracellulair calcium, en dat calcium fluxen door de plasmamembraan waarschijnlijk nodig zijn voor het effect van LH op steroid productie.

In hoofdstuk 5 zijn experimenten over het mechanisme van werking van LHRH beschreven. Cyclisch AMP is waarschijnlijk niet betrokken bij de werking van LHRH, en de effecten van LHRH zijn onafhankelijk van die van LH. Voor het effect van LHRH is de aanwezigheid van extracellulair calcium nodig. De stimulering van steroid productie door LHRH werd niet geremd wanneer calcium kanalen werden geblokkeerd, en het lijkt waarschijnlijk dat onder invloed van LHRH de intracellulaire concentratie van vrij calcium stijgt doordat calcium wordt vrijgemaakt uit intracellulaire voorraden. Uit een vergelijking van de effecten van LHRH, forbolester (een specifieke activator van eiwit kinase C) en fosfolipase C op steroid productie, eiwitfosforylering en eiwitsynthese bleek dat de effecten van LHRH en fosfolipase C vergelijkbaar waren, terwijl forbol ester en LH een soortgelijk stimulerend effect hadden op eiwitfosforylering en eiwitsynthese. Op grond van deze resultaten kan worden gecon-

cludeerd dat LHRH waarschijnlijk in eerste instantie de hydrolyse van fosfolipiden stimuleert hetgeen mogelijk resulteert in de productie van metabolieten van fosfatidylinositol, welke dan de steroid productie beinvloeden. Of eiwit kinase C door LH wordt geactiveerd, moet nog verder worden onderzocht.

In hoofdstuk 6 is het onderzoek naar de aanwezigheid en de functionele eigenschappen van de stimulerende (N_s) en remmende (N_i) guanine nucleotide bindend eiwitten in Leydig cellen beschreven. N_s en N_i zijn waarschijnlijk betrokken bij de regulatie (stimulatie en remming) van adenylaat cyclase in veel cel typen. Experimenten over de ADP-ribosylering van eiwitten met behulp van cholera toxine en pertussis toxine lieten zien dat de identieke α-subeenheid van N_s en N_i aanwezig is in Leydig cellen. Een 24 uurs preincubatie met cholera toxine of pertussis toxine resulteerde in een stimulering van de basale en de LH-gestimuleerde steroid productie. Op grond van deze resultaten is het waarschijnlijk dat N_s betrokken is bij het effect van LH op adenylaat cyclase en dat N_i ook een rol speelt bij de regulatie van steroid productie.

In hoofdstuk 7 wordt een mogelijk model van de regulatie van steroid productie in Leydig cellen gepresenteerd en bediscussieerd. Binding van LH aan membraanreceptoren resulteert in activering van N_s, dat adenylaat cyclase stimuleert. Het product van adenylaat cyclase, cyclisch AMP, activeert cyclisch AMP-afhankelijk eiwit kinase. LH stimuleert ook een influx van calcium door de plasmamembraan, mogelijk via activering van N_i. LHRH zou het metabolisme van fosfatidylinositol kunnen stimuleren, waarbij het product inositoltrifosfaat calcium ionen kan vrijmaken uit intracellulaire voorraden. De rol van de andere producten van het fosfatidylinositol metabolisme, diacylglycerol en arachidonzuur metabolieten, is niet duidelijk. Omdat zowel LH als LHRH de intracellulaire calcium concentratie verhogen, terwijl de hormonen onafhankelijk van elkaar hun werking verrichten, wordt voorgesteld dat de effecten van calcium via verschillende intracellulaire compartimenten verlopen. Calcium activeert eiwit kinases en reguleert andere functies in de cel waarschijnlijk via calcium-bindende eiwitten. De gecombi-

neerde acties van de genoemde boodschappers (cyclisch AMP, fosfatidylinositol metabolieten en calcium) resulteert in de activering van CSCC via stimulering van eiwitfosforylering, en/of eiwitsynthese, waardoor een verhoging van het aanbod van cholesterol of een directe activering van het enzym plaatsvindt.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 24 mei 1955 geboren in Volkel (gemeente Uden). Na het behalen van het Atheneum B diploma aan het Titus Brandsma Lyceum te Oss in 1974, werd in hetzelfde jaar begonnen met de studie Moleculaire Wetenschappen aan de Landbouwhogeschool in Wageningen. Tijdens de praktijktijd werd onderzoek verricht op het Department of Physiological and Environmemtal Studies (dr. D. Grierson) aan de Agricultural School van de University of Nottingham. In juni 1982 werd het ingenieursdiploma behaald met als vakken Biochemie (prof. dr. C. Veeger), Plantenfysiologie (prof. dr. J. Bruinsma) en Kolloidchemie (prof. dr. J. Lyklema). Van 16 augustus 1982 tot 1 februari 1986 was hij werkzaam als wetenschappelijk assistent op de afdeling Biochemie II (Chemische Endocrinologie) van de Faculteit der Geneeskunde van de Erasmus Universiteit Rotterdam alwaar het in dit proefschrift beschreven onderzoek werd verricht.

APPENDIX PAPER 1

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IS cAMP THE OBLIGATORY SECOND MESSENGER IN THE ACTION OF LUTROPIN ON LEYDIG CELL STEROIDOGENESIS ?

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Two adenylate cyclase inhibitors: 9-(tetrahydro-2-furyl)adenine and 2'5'-dideoxyadenosine decreased cAMP levels in LH-stimulated immature rat Leydig cells by 20-40%, independent of the concentration of LH. Steroid production was not correlated with this decrease in cAMP, but was increased (146%). The phorbol ester 4β-phorbol-12-myristate-13-acetate stimulated steroidogenesis and the phosphorylation of a 17 kD and a 33 kD protein, which was also stimulated by LH, whereas the inactive phorbol ester 4β-phorbol-12,13-diacetate did not have any effects. Moreover, the Ca^{2+}-channel blocker diltiazem inhibited LH effects, but had no direct effects on the cholesterol side chain cleavage enzyme. It is concluded that cAMP may not be the only second messenger in LH action, and that other second messenger systems are probably also involved.

Incubation of testicular Leydig cells with LH, cholera toxin or forskolin results in increased levels of cAMP (1-5), binding of cAMP to PK-A (6), activation of PK-A (7,8), protein phosphorylation (9,10) and finally in an increase in steroid production. Administration of analogues of cAMP such as dibutyryl-cAMP and 8-Bromo-cAMP to Leydig cells also results in a stimulation of steroid production (1,9). On basis of these results it is generally accepted that cAMP and specific protein phosphorylation is important in the transmission of the hormonal signal to the cholesterol side-chain cleavage enzyme in the mitochondrion after binding of LH to the receptor.

Abbreviations:

PMA: 4β-phorbol-12-myristate-13-acetate; PA: 4β-phorbol-12,13-diacetate; PK-A: cAMP dependent protein kinase; PK-C: phospholipid dependent, Ca^{2+}-activated protein kinase; LH: lutropin (luteinizing hormone); LHRHa: luliberin (lutropin releasing hormone)-analogues; PtdIns: phosphatidylinositol; EGF: epidermal growth factor; DDA: 2',5' dideoxyadenosine; TFA: 9-(tetrahydro-2-furyl)adenine.

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Several discrepancies have been observed between hormone-induced dose-response curves for cAMP and for steroid production (2,3,11). This has been explained frequently by assuming that hormone-induced changes in cAMP levels occur in specific intracellular pools of cAMP. However, transduction of hormonal signals in cells can occur also independent of cAMP and may involve PK-C (12), increased PtdIns-turnover (13) or via direct activation of a receptor linked tyrosine kinase (e.g. EGF) (14). Also, in Leydig cells LHRHa can stimulate steroid production without apparent changes in cAMP production (15,16), but the exact mechanism of action of LHRHa on these cells is unknown.

We have investigated whether cAMP has an obligatory role in the regulation of steroid production of Leydig cells by measuring the effects of inhibitors of adenylate cyclase on steroid production. The effects of phorbol ester on steroid production and specific protein phosphorylation were studied to investigate the possible involvement of PK-C in the regulation of specific protein phosphorylation and steroidogenesis. The relative importance of LH-induced fluxes of Ca^{2+} through the cell membrane in LH action was investigated using the Ca^{2+}-channel blocker diltiazem.

METHODS

Leydig cells were isolated from testes of immature (21-24 day old) Wistar rats (substrain R-Amsterdam) essentially as described by Rommerts et al. (17). Approximately 0.5×10^6 cells were incubated at 32°C under 5% CO_2 and 95% air in plastic Costar Multiwell dishes containing culture medium prepared from modified Eagle's Medium with Earle's liquid and non-essential amino acids (Gibco) containing extra glutamine (0.6 mg/ml) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, 0.6 µg/ml Fungizone) (MEM) with 1% (v/v) fetal calf serum. After one hour the floating cells were removed by washing, and the attached cells were incubated in MEM with 0.1% (w/v) BSA for one hour when effects of DDA (PL-Biochemicals Pharmacia) or TFA (SQ22.536, Squibb Institute of Medical Research,USA) were studied, or three hours in the other experiments. Pregnenolone production was measured in the presence of inhibitors of pregnenolone metabolism 20 µM SU-10603 (Ciba-Geigy) and 5 µM cyanoketone (Stirling-Winthrop) in the culture medium using a radioimmunoassay method as previously described (18). The hormone independent activity of the cholesterol side chain cleaving enzyme was measured with 25-hydroxycholesterol to evaluate whether additions had non-specific effects (19). For determination of cAMP the cells were lysed with 5% perchloric acid. The cell lysate was treated with 0.5 M Tris containing KOH sufficient to neutralize the perchloric acid, stored at -20°C and cAMP was measured as described by Schumacher et al. (5) and

Tovey et al. (20). Protein phosphorylation in intact cells was estimated as described by Bakker et al. (9).

RESULTS AND DISCUSSION

Isolated Leydig cells from immature rats were incubated with increasing concentrations of LH in the presence of an inhibitor of adenylate cyclase TFA (21-23) (Fig.1). Steroid production by Leydig cells was increased 10-30 fold by 1 ng/ml LH without detectable changes in cAMP levels, neither could effcts of TFA be demonstrated at 1 ng/ml LH. The elevated cAMP levels at higher doses of LH were decreased to 78% $\pm$ 7% by TFA, independent of LH concentrations between 5 and 100 ng/ml, whereas pregnenolone production at these concentrations of LH was stimulated to 146% $\pm$ 7%. The constant decrease in cAMP levels at LH concentrations between 5 and 100 ng/ml shows that the effect of TFA on adenylate cyclase is independent of the extent of activation of the enzyme. By extrapolation it was inferred that the same inhibition may occur at low concentrations of LH which do not cause measurable changes in cAMP. Inhibition of adenylate cyclase without a decreased, but instead a increased steroid production was also obtained with another inhibitor of adenylate cyclase: DDA (24-26) (fig. 2).

Evidence for the involvement of other transducing systems was obtained by using phorbolesters which specifically activate PK-C (27,28), and diltiazem, a Ca^{2+}-channel blocker (29). Addition of the phorbolester PMA resulted in a small stimulation of steroid production (Table 1). PMA did not have any effect on the maximally stimulating dose of LH, and the dose response curves for stimulating steroid production by LH in the absence or presence of PMA coincided (results not shown). The inactive phorbolester PA (27,28) used as an inactive control for PMA, did not have any effects on steroid production. LH and PMA both stimulated phosphorylation of a 17 kD and, although less clear, a 33 kD protein to the same extent, whereas PA had no effects on protein phosphorylation (fig. 3).

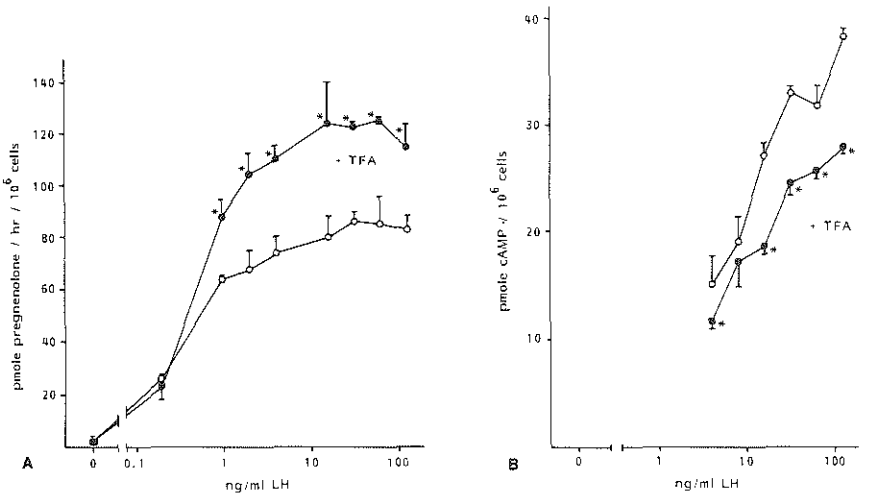

Figure 1 Effect of 9-(tetrahydro-2-furyl)adenine (TFA), an inhibitor of adenylate cyclase, on LH stimulated pregnenolone production and cAMP levels in Leydig cells. A: pregnenolone production stimulated by LH in the absence (o) or presence (●) of 100 μM TFA. B: intracellular cAMP levels stimulated by LH in the absence (o) or presence (●) of 100 μM TFA. Basal cAMP levels were 1.48 ± 0.09 (n=4) pmol/10^6 cells. LH at concentations up to 1 ng/ml did not elevate cAMP levels above the basal value. The average percentage inhibition by TFA was 22% ± 7% (SD) and independent of the concentration of LH. The values given are means ± SD (n=3). *: $P<0.01$.

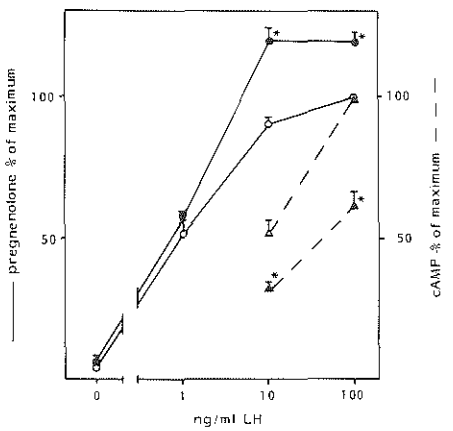

Figure 2 Effect of 2'5'-dideoxyadenosine on LH stimulated pregnenolone production and cAMP levels. Circles: pregnenolone production in the absence (o) or presence (●) of 100 μM DDA. Triangles: cAMP levels in the absence (△) or presence (▲) of 100 μM DDA. Inhibition of cAMP levels was 40% ± 3%. Values given are mean percentages ± SD (n=4), with the values at 100 ng/ml in the absence of DDA taken as 100%. *:$P<0.01$.

Table 1: EFFECT OF PHORBOL ESTERS ON LEYDIG CELL STEROIDOGENESIS

	pmol pregnenolone/h/10^6 cells	
Addition		LH (100 ng/ml)
-	1.1 ± 0.1	120.9 ± 3.2
PA (100 ng/ml)	1.7 ± 0.5	122.5 ± 8.3
PMA (100 ng/ml)	6.5 ± 1.5	120.4 ± 8.9

(means ± SD; n=3)

Diltiazem, a Ca^{2+}-channel blocker inhibited the LH stimulated pregnenolone production from endogenous cholesterol, but not the pregnenolone production in the presence of 25-hydroxycholesterol (table 2). This indicates that fluxes of Ca^{2+} may be involved in the hormonal control of endogenous steroidogenesis, but not in the cholesterol side-chain cleavage activity (19).

In this study we have shown that two inhibitors of adenylate cyclase did not inhibit, but actually stimulated the LH response. Also, we have shown that an activator of PK-C also stimulates steroidogenesis and protein

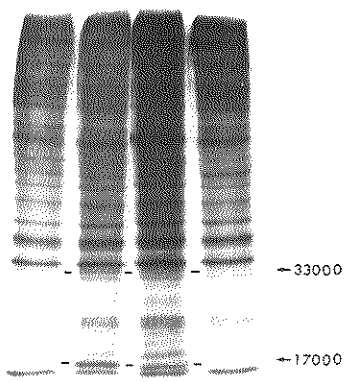

Figure 3 Protein phosphorylation pattern of Leydig cells. Lanes from left to right: control, LH, PMA, PA. Intact cells were incubated with 100 μCi $^{32}PO_4$/well for three hours, with the following additions: control, 100 ng/ml LH, 100 ng/ml PMA, 100 ng/ml PA. Cells were lysed and proteins were extracted and separated on polyacrylamide gels in the presence of sodiumdodecylsulphate as described by Bakker et al. (9).

Table 2: EFFECTS OF DILTIAZEM ON LEYDIG CELL STEROIDOGENESIS

	pmol pregnenolone/h/10^6 cells	
Addition	-	Diltiazem (100 μM)
-	2.3 ± 0.3	2.3 ± 0.1
LH (100 ng/ml)	49.0 ± 0.1	31.2 ± 0.4
25-hydroxycholesterol (30 μM)	36.1 ± 5.3	36.2 ± 0.5

(means ± SD; n=3)
Diltiazem (100 μM) was present for three hours, 25-hydroxycholesterol (30 μM) and LH (100 ng/ml) were added after the second hour.

phosphorylation, and that an inhibitor of Ca^{2+}-fluxes inhibits the LH dependent steroid production.

The absence of inhibition of steroid production by the inhibitors of adenylate cyclase could also be explained by the presence of an excess of intracellular cAMP more than required for stimulation of steroidogenesis. However, in blood platelets, it was shown that TFA could antagonise the effect of PGE_1 on blood platelet aggregation in a dose-related manner (22). Also, Fain et al. (30) found, using the adenylate cyclase inhibitor DDA, a decrease of cAMP levels, without a concomitant inhibition of lipolysis in isolated fat cells. The increase in steroid production that was observed when inhibitors of adenylate cyclase were present, cannot be readily explained.

Based on the results presented we conclude that LH stimulation of steroid production in Leydig cells may not only involve activation of PK-A, but may also activate other transducing systems such as PK-C, the PtdIns-cycle and fluxes of Ca^{2+}. An interaction between the adenylate cyclase and the Ca^{2+}-fluxes in mast cells has been suggested by Gomperts (31). He showed that the G-protein, which couples the receptor to the adenylate cyclase, may be involved also in the activation of Ca^{2+}-channels. Evidence for interactions between the adenylate cyclase and the PK-C system has also been reported, although most reports reflect that cAMP has an inhibitory rather than a stimulatory effect on the PK-C

system (13,32-35). The steroidogenic potency of PMA alone is comparable to a dose of 0.1 ng/ml LH, and does not reach the high values obtained with a maximal dose of LH. Mukhopadhyay et al.(36) also showed with mouse Leydig cells a low stimulation by PMA, and no effect on the maximally stimulating dose of LH.

Although PMA was considerably less potent than LH in stimulating steroid production, the phorbolester and LH had similar effects on protein phosphorylation. This may indicate that either PK-C is involved in LH action on Leydig cells, or that PK-A and PK-C act more or less independently by phosphorylation of proteins at different sites. Both interpretations support however that the different transducing systems act on the same 'target proteins'. Furthermore, the low potency of PMA in stimulating steroid production in comparison with the efficient stimulation of protein phosphorylation, suggests that in addition to PK-C other transducing systems such as cAMP and fluxes of Ca^{2+} are required for the stimulation of steroidogenesis by LH.

The possibility that LH stimulates different messenger systems may cast new light on old observations. Thusfar the stimulation of steroid production by low doses of LH without measurable changes in cAMP has been explained generally by assuming that elevations of cAMP levels take place in small intracellular pools but that such changes escape detection because they are masked by the large cAMP pools which are not affected by LH. It is difficult to envisage such isolated pools, when it is known that cAMP can pass the cell membrane, and can accumulate in the extracellular space (5,37). An alternative explanation is that LH activates, in addition to adenylate cyclase, other messenger systems. This possibility could not be ruled out in earlier experiments, not even when a correlation was established between binding of cAMP to PK-A, or between the activity of PK-A, and steroid production (2,6,7). Involvement of other transducing systems may also explain why the ED_{50} of hCG stimulated testosterone production by Leydig cells is 60 times lower than the ED_{50} of cholera

toxin (hCG: 10^{-12}M; cholera toxin: $0.6x10^{-10}$M (2), whereas the dose response curves for stimulation of cAMP production by hCG and cholera toxin are similar.

In conclusion, the present results show that cAMP may not be the sole messenger for LH action on steroid production in Leydig cells. The criteria as originally proposed by Sutherland and Robison (38) for evaluating whether cAMP is the second messenger in the transmission of a hormonal signal can be improved by including as additional criterium the effects of inhibitors of adenylate cyclase on the physiological response.

ACKNOWLEDGMENT

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APPENDIX PAPER 2

Effects of LH and an LH-releasing hormone agonist on different second messenger systems in the regulation of steroidogenesis in isolated rat Leydig cells

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ABSTRACT

The stimulation of steroid production in Leydig cells by LH is accompanied by increased cyclic AMP levels, activation of protein kinase A, increased phosphorylation of at least six phosphoproteins and requires protein synthesis. However, an LH-releasing hormone agonist (LHRH-A) can stimulate steroid production without stimulation of cyclic AMP levels. In the present study we have shown that LH action involves calcium fluxes through the plasma membrane, in addition to activation of protein kinase A. The action of LHRH-A, in contrast, does not require calcium fluxes and is not potentiated by 1-methyl-3-isobutylxanthine, indicating that cyclic AMP is not involved. Extracellular calcium is required for the action of both LH and LHRH-A. An increase in intracellular calcium concentration due to the effect of ionophore A23187 did not stimulate steroidogenesis and had deleterious effects on intracellular adenosinetriphosphate levels. LH and 4β-phorbol-12-myristate-13-acetate (PMA), an activator of protein kinase C, both stimulated phosphorylation of proteins of 17 000 and 33 000 mol. wt, whereas LHRH-A had no effect. However, compared with the effect of LH, PMA had a much smaller effect on steroid production, indicating that even if protein kinase C may be activated by LH its role in the regulation of steroid production may be less important than the role of protein kinase A. Action of LHRH-A does not appear to be mediated by calcium fluxes, protein kinase C activation or active protein phosphorylation. *J. Endocr.* (1986) **108**, 431–440

INTRODUCTION

It is generally accepted that cyclic AMP (cAMP) plays an important role in the regulation of steroid production, since stimulation of the mitochondrial cholesterol side-chain cleaving enzyme (CSCC) by luteinizing hormone (LH) is accompanied by increased levels of cAMP, activation of protein kinase A (PK-A) and increased phosphorylation of at least six proteins (Cooke, Lindh & Janszen, 1976; Podesta, Dufau, Solano & Catt, 1978; Bakker, Hoogerbrugge, Rommerts & van der Molen, 1983). However, other second messenger systems, not linked to cAMP, have also been implicated in the action of LH on CSCC activity. Blocking calcium channels with cobalt inhibited the effect of LH (Moger, 1983), and the introduction of Ca^{2+}-calmodulin complexes into Leydig cells via liposomes stimulated CSCC activity (Hall, Osawa & Mrotek, 1981).

In addition to second messenger systems which may be triggered by LH, other LH-independent mechanisms for the regulation of steroid production in Leydig cells exist; e.g. epidermal growth factor (Ascoli, 1981; Melner, Lutin & Puett, 1982), LH-releasing hormone (LHRH) (Hsueh & Jones, 1981; Sharpe, 1982, 1984) and vasopressin (Meidan & Hsueh, 1985), but their mechanism of action in unknown. Of these, the action of LHRH and its analogues has been investigated most. After prolonged administration *in vivo* in hypophysectomized rats, LHRH agonists reduce testicular LH receptors and testosterone secretion (Bambino, Schreiber & Hsueh, 1980; Cao, Sundaram, Bardin *et al.* 1982). These direct effects are mediated by specific high affinity receptors for LHRH on Leydig cells

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(Sharpe & Fraser, 1980). Studies with isolated Leydig cells show that the effects of LHRH agonists on Leydig cell steroidogenesis are biphasic. In short-term incubations (< 3 h) LHRH agonists stimulate steroid production, but when cells are incubated for 24 h or longer with LHRH agonists, the LH-dependent steroidogenesis is inhibited by the releasing hormone (Hunter, Sullivan, Dix *et al.* 1982; Browning, D'Agata, Steinberger *et al.* 1983).

The mechanism of LHRH action on Leydig cell steroidogenesis is not clear. Results from different workers indicate that LHRH action is not mediated by cAMP, and it has been postulated that calcium is involved in the regulation of steroid production by LHRH (Lin, 1984; Sullivan & Cooke, 1984*a*). However, there are many steps between the binding of LH to the receptor and the stimulation of CSCC activity, and the possible locus of the calcium effect is still unknown.

Calcium-dependent, phospholipid-activated protein kinase (PK-C) has been implicated in LHRH action on pituitary gonadotrophs (Hirota, Hirota, Aguilera & Catt, 1985). PK-C appears to be present in pig Leydig cells (Kimura, Katoh, Sakurada & Kubo, 1984), and phosphatidylinositol breakdown, which may lead to activation of PK-C, was stimulated by an LHRH agonist in rat Leydig cells (Molcho, Zakut & Naor, 1984). However, there were no indications of a possible effect of LH or an LHRH agonist on calcium fluxes or PK-C in Leydig cells.

We have therefore investigated the relative importance of cAMP, PK-C, calcium fluxes and protein phosphorylation in the LH- and LHRH agonist-mediated activation of steroid production in rat Leydig cells.

MATERIALS AND METHODS

Isolated cells were incubated in modified Eagle's medium with Earle's salts and non-essential amino acids containing 100 μg streptomycin/ml, 0·6 μg fungizone/ml, 100 i.u. penicillin/ml (MEM) and 1% (v/v) fetal calf serum (Gibco, Grand Island, NY, U.S.A.) (MEM/FCS) or 0·1% (w/v) bovine serum albumin (BSA) (MEM/BSA). In some experiments modified Krebs–Ringer buffer with 0·2% (w/v) glucose, 0·1% (w/v) BSA (pH 7·4), containing various low concentrations of calcium was used. The inclusion of ethanedioxybis(ethylamine)tetra-acetic acid (EGTA; 0·1 mmol/l) in the culture medium assured the virtual absence of any calcium during incubations.

Ovine LH (NIH-LH-S18; 1·03 i.u/mg) was a gift from the Endocrinological Study Section of the National Institute of Health, Bethesda, MD, U.S.A. [D-Ser(Bu^t)^6, des Gly^{10}-NH$_2$]LHRH-ethylamide (HOE 766, an LHRH agonist; LHRH-A) and Ac-(D)pClPhe-(D)pClPhe-(D)Trp-Ser-Tyr-(D)Phe-Leu-Arg-Pro-(D)Ala-NH$_2$ (ORG30093, an LHRH antagonist) were kindly provided by Hoechst Pharma (Amsterdam, The Netherlands) and Organon (Oss, The Netherlands) respectively. Diltiazem, 1-methyl-3-isobutylxanthine (MIX), collagenase, 4β-phorbol-12-myristate-13-acetate (PMA), 4β-phorbol-13-monoacetate (PA) (Sigma, St Louis, MO, U.S.A.), 5-cholesten-3β,25-diol (25-hydroxycholesterol) (Steraloids, Wilton, NH, U.S.A.) and A23187 (Boehringer, Mannheim, F.R.G.) were used at the concentrations indicated. Carrier-free $^{32}PO_4$ was from Amersham International plc, Bucks. 2α-Cyano-17β-hydroxy-4,4′, 17α-trimethyl-5-androsten-3-one (cyanoketone) and 7-chloro-3,4-dihydro-2(3-pyridyl)-1-(2H)-naphthalenone (SU-10603) were kindly donated by Dr R. Neher of the Friedrich Miescher Institute, Basle, Switzerland.

Immature rats from a Wistar substrain (R-Amsterdam) were used. Leydig cells were obtained through collagenase treatment of decapsulated testes from 21- to 24-day-old animals as described by Rommerts, Molenaar & van der Molen (1985). The cells were allowed to attach to Costar multiwell dishes in MEM/FCS. After 1 h the floating cells were removed by washing, and the cells attached to the dishes were used for the experiments. Incubations were carried out in MEM/BSA at 32 °C (5% CO_2 in air) in the presence of the pregnenolone metabolism inhibitors cyanoketone (5 μmol/l) and SU-10603 (19 μmol/l) (van der Vusse, Kalkman & van der Molen, 1974). At the end of the incubation the medium was collected for determination of pregnenolone by radioimmunoassay (van der Vusse, Kalkman & van der Molen, 1975). The smallest amount that could be measured was 5–10 pmol pregnenolone, the intra-assay variation was in the order of 20%, and the recovery of added pregnenolone was 107 ± 11%. For the determination of cAMP and adenosinetriphosphate (ATP) the cells were lysed with 5% perchloric acid at 4 °C directly after removal of the medium. The lysate (200 μl) obtained was mixed with Tris buffer (1·1 mol/l, 20 μl), adjusted to pH 7 with KOH, stored at 20 °C and assayed for cAMP using a cAMP assay kit (Amersham International plc; smallest amount measurable, 0·5 pmol; intra-assay variation 11%) or for ATP as described by Grootegoed, Jansen & van der Molen (1984). In some experiments pregnenolone production in the presence of 25-hydroxycholesterol (30 μmol/l) was measured. This production can be taken as a measure of the CSCC activity and is used to give an indication of non-specific effects of various additions to the incubations (Mason & Robidoux, 1978; Brinkmann, Leemborg, Rommerts & van der Molen, 1984).

Protein phosphorylation was measured in intact

cells. Cells (10^6) were incubated for 3 h in 0·5 ml Krebs–Ringer buffer without phosphate but containing 0·2% (w/v) glucose and 0·1% (w/v) BSA, in the presence of 37 MBq $^{32}PO_4$. The proteins were extracted and separated on polyacrylamide gradient gel electrophoresis in the presence of sodiumdodecylsulphate (8–15% polyacrylamide gradient) as described before (Bakker, Hoogerbrugge, Rommerts & van der Molen, 1981). The gels were dried and autoradiographed using Kodak SB-5 X-ray film. Molecular weight was determined using an electrophoresis calibration kit from Pharmacia Fine Chemicals (Uppsala, Sweden).

The results are given as means ± S.D. of four or more observations obtained using two or more different cell preparations. Each observation is the result of an assay performed in duplicate. The significance of differences between results was calculated using Student's *t*-test for non-paired data. Differences were considered to be significant when *P* values were less than 0·01.

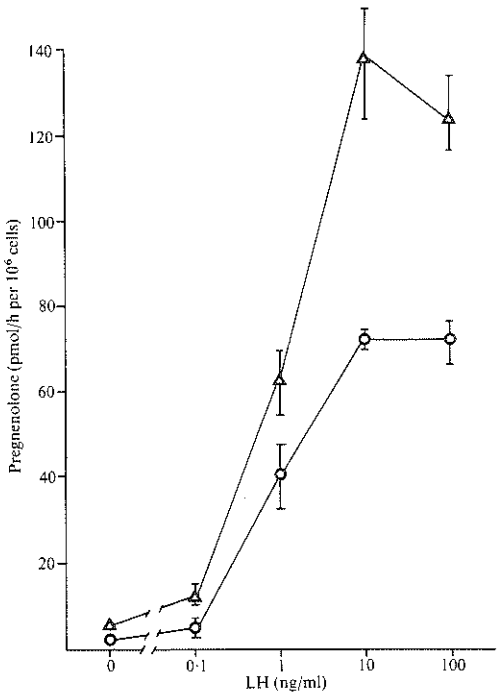

FIGURE 1. Effect of LH-releasing hormone agonist (LHRH-A) on LH-stimulated pregnenolone production by Leydig cells from immature rats. Leydig cells were incubated for 3 h with different concentrations of LH in the absence (○) or presence (△) of LHRH-A (40 nmol/l). Values are means ± S.D. ($n = 4$) of two different cell preparations. All values in the presence of LHRH-A are significantly ($P < 0·01$) different from corresponding productions with LH alone (Student's *t*-test).

RESULTS

Steroid production

Leydig cells attached to the plastic surface of culture dishes were incubated for 3 h in the presence of LH (100 ng/ml) or in the presence of LH (100 ng/ml) and LHRH-A (40 nmol/l) (Fig. 1). Pregnenolone production was stimulated 35-fold when cells were incubated with LH alone compared with controls. The response could be further enhanced (60-fold) when LHRH-A (40 nmol/l) was added together with LH (Fig. 1). In the presence of LH, the addition of LHRH-A caused an additional steroid production of 60 pmol/h per 10^6 cells; without LH the increase in pregnenolone production was only 8 pmol/h per 10^6 cells. LHRH-A alone stimulated pregnenolone production in a dose-responsive manner, with a dose for half-maximal stimulation of 0·1 nmol/l (Fig. 2). Cells cultured for 48 h in the absence of LH or LHRH-A could still be stimulated with LH (control, 8 ± 1; LH, 145 ± 12 pmol/h per 10^6 cells: $n = 8$ of three different cell preparations), but not with LHRH-A. The effect of LH-stimulated pregnenolone production by cells cultured for 48 h in the presence of both LH and LHRH-A was less than the effect of LH on cells after

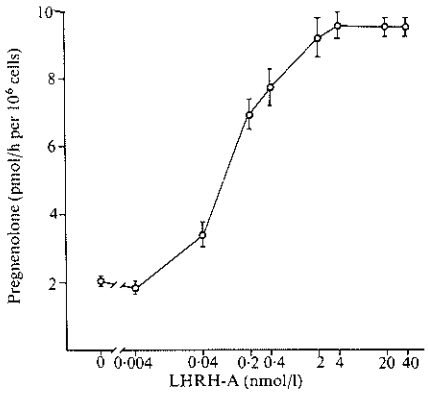

FIGURE 2. Effect of LH-releasing hormone agonist (LHRH-A) on pregnenolone production by immature rat Leydig cells. Cells were incubated from 3 h in the presence of various concentrations of LHRH-A. Values are means ± S.D. ($n = 4$) of two different cell preparations. All productions in the presence of LHRH-A, except the value at 0·004 nmol LHRH-A/l, were significantly ($P < 0·01$) different from the control (Student's *t*-test).

culture with LH alone (180 ± 4 compared with 105 ± 3 pmol/h per 10^6 cells: $n = 8$ of three different cell preparations). The effect of LH on cells after culture for 48 h in the presence of LHRH-A did not differ from the effect of LH on control cells. Both the stimulatory (Figs 1 and 2) and the inhibitory effects of LHRH-A were completely abolished when the LHRH antagonist (400 nmol/l) was present during the incubations or cultures. The LHRH antagonist did not have any effect by itself.

Role of cAMP

The role of cAMP in the mechanism of action of LHRH-A was investigated by measuring the effect of LH and LHRH-A on intracellular cAMP levels, and by comparing the effect of the phosphodiesterase inhibitor MIX on steroid production stimulated by LHRH-A and LH (Table 1). Luteinizing hormone (100 ng/ml) enhanced intracellular cAMP levels threefold and a further increase was obtained when, in addition to LH (100 ng/ml), MIX (200 μmol/l) was added to the incubation medium. Under these conditions MIX had no effect on steroid production. In cells incubated with a lower concentration of LH (0·2 ng/ml) MIX had no effect on cAMP levels, but it stimulated pregnenolone production approximately fourfold. Neither intracellular cAMP levels nor pregnenolone production in the presence of LHRH-A was affected by the addition of MIX.

Role of calcium

Effects of changes in the calcium concentration on steroid production were investigated in different ways: (1) by lowering the extracellular calcium concentration, (2) by blocking the calcium channels with the calcium channel blocking agent diltiazem, and (3) by increasing the calcium flux through the plasma membrane with the calcium ionophore A23187. In all experiments pregnenolone production in the presence of 25-hydroxycholesterol (30 μmol/l) was also measured and taken as a measure of CSCC activity (Mason & Robidoux, 1978; Brinkmann *et al.* 1984).

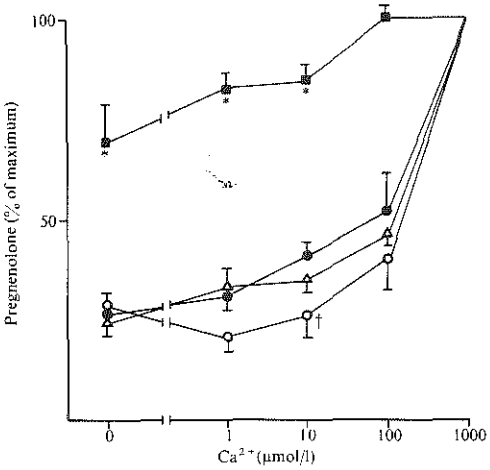

FIGURE 3. Effect of extracellular calcium concentration on hormone- and 25-hydroxycholesterol-stimulated steroid production. Cells were incubated for 3 h in the presence of LH (100 ng/ml, ●), LH (0·2 ng/ml, △), LH-releasing hormone agonist (LHRH-A; 40 nmol/l, ○) or 25-hydroxycholesterol (30 μmol/l, ■). Values are mean percentages ± S.D. ($n = 4$) of two different cell preparations, with values at 1 mmol Ca^{2+}/l taken as 100%. Pregnenolone production (pmol/h per 10^6 cells) at 1 mmol Ca^{2+}/l was: LH (100 ng/ml) 116·0; LH (0·2 ng/ml) 20·8; LHRH-A (40 nmol/l) 12·3; 25-hydroxycholesterol (30 μmol/l), 120·6. *$P < 0.01$ compared with 25-hydroxycholesterol-stimulated production at 1 mmol Ca^{2+}/l. †$P < 0.01$ compared with LH-stimulated production at 10 μmol Ca^{2+}/l. All other LHRH-A- and LH-stimulated productions at identical Ca^{2+} concentrations were not significantly different (Student's *t*-test).

TABLE 1. Effects† of 1-methyl-3-isobutylxanthine (MIX; 200 μmol/l) on cyclic AMP (cAMP) levels and pregnenolone production in rat Leydig cells in the presence of LH and LH-releasing hormone agonist (LHRH-A). Values are means ± S.D. ($n = 4$) of two cell preparations

	Pregnenolone (pmol/h per 10^6 cells)		**cAMP** (pmol/10^6 cells)	
	No MIX	MIX	No MIX	MIX
Additions				
None	1·6 ± 0·3	6·0 ± 1·6*	7·2 ± 2·2	6·3 ± 1·8
LH (100 ng/ml)	80·9 ± 3·5	79·6 ± 14·5	21·9 ± 2·9	36·1 ± 7·4*
LH (0·2 ng/ml)	18·6 ± 5·1	87·2 ± 12·6*	6·0 ± 3·4	7·7 ± 1·8
LHRH-A (40 nmol/l)	13·3 ± 3·8	18·6 ± 8·5	7·3 ± 3·9	7·9 ± 2·3

*$P < 0.01$ vs corresponding values without MIX (Student's *t*-test).
†Leydig cells were incubated in the presence of the additions as indicated. After 3 h the medium was collected for pregnenolone measurement and the cells were lysed for cAMP determination.

After lowering extracellular calcium from 1 mmol to 1 μmol/l steroidogenic activation in the presence of LHRH-A and different concentrations of LH was inhibited to approximately 30% of the control values (Fig. 3). Inhibition of the 25-hydroxycholesterol-dependent pregnenolone production at calcium concentrations lower than 10 μmol/l indicated that at these concentrations mitochondrial CSCC activity was probably directly affected.

Blocking the calcium influx with diltiazem inhibited the LH-stimulated steroid production by 27% both at 0·1 and 100 ng/ml, whereas the LHRH-A-stimulated steroid production was not affected. Steroid production in the presence of 25-hydroxycholesterol was not inhibited (Fig. 4).

Treatment of the cells with different concentrations of A23187 inhibited the effect of LH (100 ng/ml) and LHRH-A (40 nmol/l), but not the effect of LH (0·2 ng/ml) on pregnenolone production (Fig. 5). Basal production was not affected. Steroidogenesis in the presence of 25-hydroxycholesterol was also inhibited at low concentrations of A23187, indicating that the ionophore had direct effects on mitochondrial CSCC. The inhibition of CSCC might have been caused by cell damage, and therefore intracellular ATP levels were measured. After incubation of the cells for 3 h in the presence of different concentrations of A23187 the following ATP levels were measured: control, 2·0 ± 0·1; A23187 (0·1 μmol/l), 1·0 ± 0·2 ($P < 0·01$);

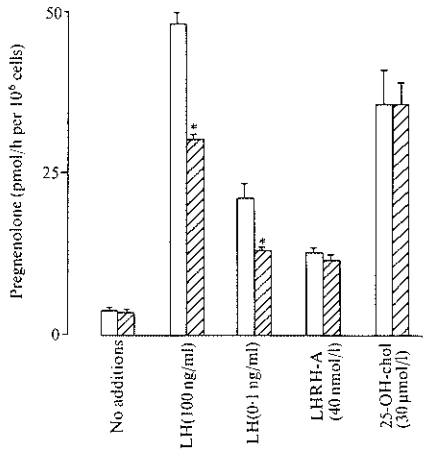

FIGURE 4. Effects of the calcium-channel blocker diltiazem (hatched bars) on pregnenolone production by immature rat Leydig cells in the presence of LH, LH-releasing hormone agonist (LHRH-A) and 25-hydroxycholesterol (25-OH-chol). Cells were incubated for 3 h with the hormone concentrations indicated in the absence or presence of diltiazem (100 μmol/l). Controls are shown by open bars. Values are means ± S.D. ($n = 4$) of two cell preparations. *$P < 0·01$ compared with corresponding incubation without diltiazem (Student's t-test).

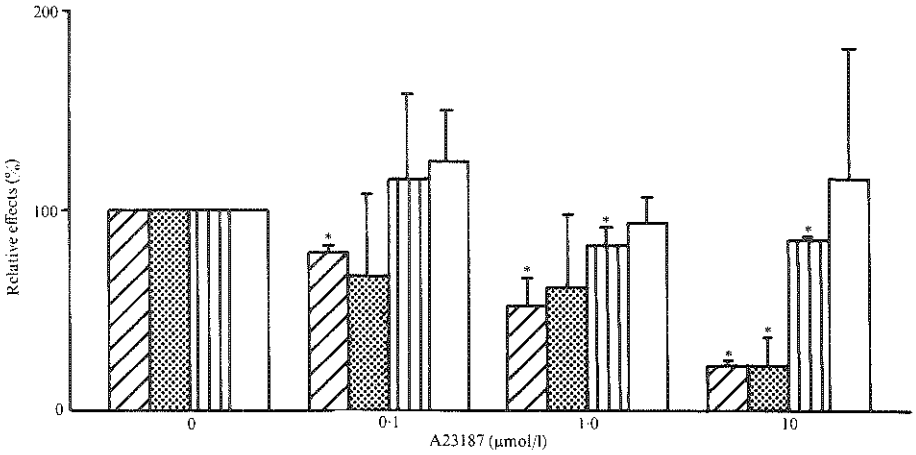

FIGURE 5. Effects of calcium ionophore A23187 on pregnenolone production by immature rat Leydig cells in the presence of LH (100 ng/ml; cross-hatched bars), LH (0·2 ng/ml; vertical-hatched bars), LH-releasing hormone agonist (LHRH-A) (40 nmol/l; open bars) and 25-hydroxycholesterol (30 mol/l; stippled bars). Values are mean percentages ± S.D. ($n = 4$) of two different cell preparations, with the values in the absence of A23187 taken as 100%. Pregnenolone productions in the absence of A23187 (pmol/h per 10^6 cells): LH (100 ng/ml) 91·2; LH (0·2 ng/ml) 14·7; LHRH-A (40 nmol/l) 9·7; 25-hydroxycholesterol (30 μmol/l) 121·2. *$P < 0·01$ compared with corresponding control (Student's t-test).

A23187 (1 μmol/l), 0·5 ± 0·1 nmol ATP/10^6 ($P < 0·01$) cells (means ± S.D.) $n = 3$, one cell preparation). These results show that ATP levels were progressively decreased after incubation of the cells with increasing concentrations of A23187.

Role of protein kinase C

To investigate whether LHRH-A acts through the PK-C system, the effect of a specific activator of PK-C, the phorbol ester PMA (Castagna, Takai, Kaibuchi *et al.* 1982; Kikkawa, Takai, Tanaka *et al.* 1983) on pregnenolone production was measured. In 3-h incubations PMA (100 ng/ml) alone stimulated steroid production approximately threefold (control, 2·0 ± 0·1; PMA, 5·9 ± 0·2 pmol pregnenolone/h per 10^6 cells) (Fig. 6). However, whereas LHRH-A potentiated LH action on pregnenolone production, PMA inhibited pregnenolone production in the presence of low concentrations of LH. The maximal steroid production obtained with high doses of LH was not inhibited by PMA.

The phorbol diester PA (100 ng/ml) had no effect on steroid production (results not shown).

Protein phosphorylation

The pattern of protein phosphorylation was investigated to elucidate further the second messenger systems for LHRH and LH which may play a role in the regulation of steroid production in the Leydig cell (Fig. 7). Both PMA and LH stimulated the phosphorylation of a protein of 17000 mol. wt and of 33000 mol. wt, whereas LHRH-A and PA (100 ng/ml) did not have an effect on protein phosphorylation.

DISCUSSION

The involvement of different second messenger systems in the control of steroid production by LH and LHRH was investigated in Leydig cells from immature rats. The present study confirms that cAMP is not involved in the action of LHRH on Leydig cell steroid production and that calcium may be important, and extends these observations: extracellular calcium, but not calcium fluxes are essential for LHRH-A action. Moreover, the results indicate that LHRH-A may not activate PK-C.

After incubation of the cells for 2 days with LH and LHRH-A, the subsequent LH-stimulated steroid production was lower than the production in cells preincubated with LH alone. This might be caused by an effect of LHRH-A on cytochrome P450 enzymes as observed for microsomal enzymes by Magoffin & Erickson (1982) after incubating ovarian cells with LHRH-A. However, LHRH-A might also have acted

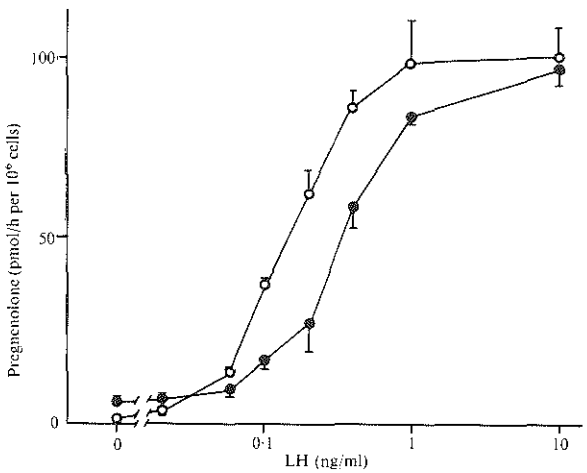

FIGURE 6. Effect of phorbol ester 4β-phorbol-12-myristate-13-acetate (PMA) on LH-stimulated pregnenolone production by immature rat Leydig cells. Cells were incubated for 3 h with different concentrations of LH in the absence (○) or presence (●) of PMA (100 ng/ml). Values are means ± S.D. ($n = 4$) of two different cell preparations. All values in the presence of PMA were significantly ($P < 0·01$) different from the corresponding value without PMA, except the value at 10 ng LH/ml (Student's *t*-test).

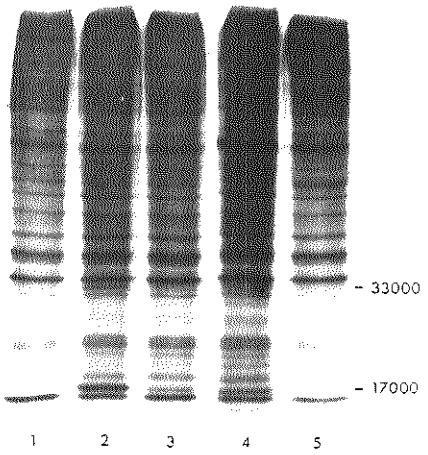

FIGURE 7. Protein phosphorylation pattern of immature rat Leydig cells. Cells were incubated alone or with either LH (100 ng/ml), 4β-phorbol-12-myristate-13-acetate (PMA; 100 ng/ml), 4β-phorbol-13-monoacetate (PA; 100 ng/ml) or LH-releasing hormone agonist (LHRH-A; 40 nmol/l) in the presence of $^{32}PO_4$. Proteins were extracted and separated on 8–15% polyacrylamide gel electrophoresis in the presence of sodiumdodecylsulphate. The autoradiograph of the dried gel is shown. Lane 1, control; lane 2, LH; lane 3, LHRH-A; lane 4, PMA and lane 5, PA.

on steps between binding of LH to the receptor and activation of the mitochondrial CSCC. Down-regulation of LH receptors appears to be unlikely because a 60–80% receptor loss, induced by injection of human chorionic gonadotrophin (hCG), did not cause a decrease of hCG-dependent pregnenolone production in intact rats (Cigorraga, Dufau & Catt, 1978). LHRH-A had long-term inhibitory actions when LH was also present in the preincubation, but enhanced the stimulation of pregnenolone production by LH in short-term incubations. This synergistic effect of LHRH-A on LH-stimulated pregnenolone production suggests that LHRH-A employs a specific pathway independent of LH. The time-related negative or positive effect of LHRH-A on LH-induced steroid production could be important for the physiological function of LHRH or LHRH-like molecules and suggestions have been made that LHRH-like molecules produced by Sertoli cells could exert paracrine effects on Leydig cells (Sharpe, Fraser, Cooper & Rommerts, 1981; Sharpe, 1982). However, there is no direct evidence for the possible testicular production of LHRH (Verhoeven & Cailleau, 1985).

Whatever the physiological function of LHRH or LHRH-like factors in the testis may be, the mechanism of LHRH action is intriguing because cAMP appears not to play an important role, since LHRH-A does not enhance cAMP levels (Lin, 1984; Sullivan & Cooke, 1984*b*). However, the absence of increased cAMP levels does not necessarily mean that cAMP is not involved, since low concentrations of LH can cause a large steroidogenic response without a concomitant measurable increase in cAMP levels (Cooke *et al.* 1976; Podesta *et al.* 1978). Our experiments with MIX show that inhibition of phosphodiesterase can enhance the effect of 0·2 ng LH/ml on pregnenolone production four-to fivefold, and a maximal steroid production is obtained without a measurable rise in cAMP levels. Since MIX inhibits cAMP breakdown, this result shows that cAMP is involved in LH action. MIX did not influence the LHRH-A action on steroid production indicating that cAMP is probably not involved in the action of LHRH-A. Sullivan & Cooke ((1984*b*) have observed inibition of LH-stimulated cAMP levels by LHRH-A, whereas LHRH-A stimulated LH-dependent steroid production. We have observed a similar discrepancy between decreased cAMP levels and an increase in steroid production after inhibition of adenylate cyclase (Themmen, Hoogerbrugge, Rommerts & van der Molen, 1985). These observations suggest that, in addition to cAMP, other second messenger systems could be involved in the action of LH. We therefore investigated the possible involvement of calcium and PK-C in LH and LHRH-A action.

A decrease in the extracellular calcium concentration resulted in a concomitant decrease of both LH- and LHRH-A-dependent steroid production. The CSCC enzyme itself, or the NADPH generation within the mitochondria, was much less affected by the low extracellular calcium concentration, as judged by the small effects on steroid production in the presence of 25-hydroxycholesterol, which is a good indication of CSCC activity (Mason & Robidoux, 1978; Brinkmann *et al.* 1984). Similar effects of low extracellular calcium concentrations have been observed in previous work studying Leydig cells (Janszen, Cooke, van Driel & van der Molen, 1976), adrenocortical cells (Trzeciak & Mathé, 1981) and granulosa cells (Veldhuis & Klase, 1982). Low extracellular calcium concentrations do not cause damage, since the inhibiting effects of low calcium concentrations on Leydig cells are reversible (Janszen *et al.* 1976). Moreover, extracellular calcium is not required for insulin action on hexose transport in L_6 muscle cells (Klip, Li & Logan, 1984). Calcium can influence the delivery of cholesterol to, or its utilization by, mitochondria in the Leydig cell (Hall *et al.* 1981). Hence, calcium ions may regulate intracellular events important for the formation of pregnenolone. Subsequently, the importance of calcium fluxes

through the plasma membrane for LH and LHRH-A action was investigated.

The inhibitory effects of diltiazem on LH-stimulated steroid production suggest that calcium fluxes through the plasma membrane are involved in LH action. Pregnenolone production in the presence of 25-hydroxycholesterol was not inhibited, which may reflect the fact that diltiazem did not affect mitochondrial CSCC. Similar results with ovarian cells have been reported by Veldhuis & Klase (1982). LHRH-A-dependent steroid production was not affected by diltiazem, suggesting that calcium fluxes through the plasma membrane are less important in LHRH-A action. It was recently reported that another channel blocker, nifedipine, inhibited LHRH-A-dependent steroid production (Lin, 1984), but in the present study the specificity of the effect of nifedipine was not evaluated. In order to obtain a more conclusive answer on the importance of calcium in LHRH-A action, direct measurement of calcium ions after stimulation of Leydig cells with LHRH-A is necessary. Preliminary experiments with the fluorescent probe Quin-2, however, showed an insufficient loading capacity for the probe of immature rat Leydig cells. Another approach to investigate the effect of artificially increased intracellular calcium concentration with calcium ionophore A23187 was therefore chosen.

Incubation of Leydig cells with A23187 inhibited basal or hormone-stimulated pregnenolone production. The ionophore has been used frequently to investigate the role of calcium ions, and biological effects (such as secretion of proteins) have been shown after application of A23187 (Zawalich, Brown & Rasmussen, 1983; Conn, 1984). Inhibition of the 25-hydroxycholesterol-dependent response indicated that A23187 directly affected the CSCC enzyme. Intracellular ATP levels of the cells were measured after incubation with A23187 to evaluate another mitochondrial function. Levels of ATP were decreased after application of A23187, suggesting that the ionophore has deleterious effects on immature rat Leydig cells. Thus results with the ionophore are inconclusive with respect to the involvement of an increase of intracellular calcium in LHRH-A action, but show that A23187 has deleterious effects in our cell system. It has been shown by Molcho *et al.* (1984) that LHRH-A can stimulate the turnover of phosphatidylinositol in Leydig cells. We have investigated whether LHRH-A could activate PK-C, since stimulation of phosphatidylinositol breakdown is thought to result in activation of PK-C, in addition to its effect on intracellular calcium release (Berridge, 1984).

PMA stimulates pregnenolone production in immature rat Leydig cells with approximately the same potency as LHRH-A, suggesting that the releasing hormone acts via PK-C. However, in contrast with LHRH-A, PMA did not potentiate LH-dependent steroid production. Moreover, the stimulatory effect of PMA on protein phosphorylation was very similar to the effect of LH, whereas LHRH-A had either no effects or minor effects, but at least much less than PMA. These results do not support the hypothesis that LHRH-A acts through PK-C.

LH and PMA both stimulate the phosphorylation of the nuclear 17000 mol. wt and the ribosomal 33000 mol. wt. protein S_6 to the same extent (Bakker *et al.* 1981), but LH is tenfold more effective than PMA in stimulation of pregnenolone production. This suggests that phosphorylation of the 17000 and 33000 mol. wt proteins alone is not sufficient for the acute stimulation of steroidogenesis by LH. The similarity in the patterns of protein phosphorylation stimulated by LH and PMA may suggest a role for PK-C in LH action in addition to cAMP-activated PK-A. However, the inhibition of steroid production by PMA observed in the presence of low doses of LH is puzzling. It may be that PK-C and PK-A, under the influence of different doses of LH, phosphorylate different sites on the same protein, as has been shown for ribosomal protein S_6 (Trevillyan, Perisic, Traugh & Byus, 1985), but a connection between phosphorylation sites and function of ribosomal protein S_6 has not been demonstrated.

From our present results we conclude that LHRH-A does not mediate its effects on steroid production through PK-A or PK-C, and that calcium fluxes through the plasma membrane do not seem to be important in LHRH action, whereas LH action may involve PK-A and PK-C activation and calcium fluxes. Both hormones require the presence of extracellular calcium. The possible role of PK-C activation in LH action, suggested by the similarity of the patterns of protein phosphorylation, remains to be elucidated.

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APPENDIX PAPER 3

COMPARISON OF THE CELLULAR COMPOSITION AND STEROIDOGENIC PROPERTIES OF INTERSTITIAL CELL PREPARATIONS ISOLATED FROM IMMATURE AND MATURE RAT TESTIS.

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Running head: interstitial cells from mature and immature rat testes

ABSTRACT

Interstitial cell preparations from both immature (21-24 days old) and mature (60-90 days old) rats are used in many investigations on the regulation of steroidogenesis. We have compared the morphological and steroidogenic properties of interstitial cell preparations isolated from testes of immature and mature rats. Collagenase dispersed cells isolated from immature rats contained 40-60% Leydig cells, 37% mesenchymal cells and no macrophages. Cell preparations from mature rats, after a Ficoll purification step, consisted of 80% Leydig cells and 20% macrophages. A 90% pure Leydig cell preparation could be obtained after further purification of the preparation isolated from immature rats with a Percoll gradient centrifugation step. Fractionation of this gradient revealed that the distribution through the gradient of steroidogenic enzymes and LH-dependent steroid production was similar. The results indicate that the steroidogenic activity per Leydig cell from mature rats is fourfold larger than the activity in immature rat Leydig cells after stimulation with LH, dibutyryl-cyclic AMP or in the presence of 22R-hydroxycholesterol.

INTRODUCTION

Leydig cells from mature rats have been used frequently for studies on the regulation of steroidogenesis by LH and other factors (Cooke & Sullivan, 1985; Adashi & Hsueh, 1982; Sharpe, 1984). After collagenase dispersion of testis tissue the isolated cell preparation generally contains approximately, 10% Leydig cells, although the recovery of intact Leydig cells from the original testis is less than 10% (Molenaar, Rommerts & van der Molen, 1983; Rommerts, Molenaar & van der Molen, 1985). The purity and viability of the isolated cell preparation can be greatly improved after density centrifugation and selection through attachment of intact cells to culture dishes (Cooke, Magee-Brown, Golding & Dix, 1981; Molenaar et al., 1983). Leydig cells from immature (21-24 days old) rats can be obtained in a rather pure state (40%) and in much higher yield (90%) after collagenase treatment of the testis, whereas almost no damaged Leydig cells are present (Molenaar et al., 1983). Isolated immature Leydig cells retain their LH-dependent steroidogenic properties in culture for 3 days

(Berns, Brinkmann, Rommerts, Mulder & van der Molen, 1985), in contrast to isolated Leydig cells from mature rats, which lose more than 90% of their steroid producing capacity in response to LH under the same incubation conditions (Hunter, Sullivan, Dix, Aldred & Cooke, 1982; Rommerts, van Roemburg, Lindh, Hegge & van der Molen, 1982). Since Leydig cells from immature rats have the advantages mentioned above compared with cells isolated from mature rats, we have used these cells for studies on the regulation of steroid production (Themmen, Hoogerbrugge, Rommerts & van der Molen, 1985, 1986).

It is known that during testicular development the Leydig cell undergoes differentiation. Morphological changes such as an increase in the amount of cells, the size of the cells and the number of LH receptors per cell have been described (Knorr, Vanha-Perttula & Lipsett, 1970; Tapanainen, Kuopio, Pelliniemi & Huhtamiemi, 1984; Clausen, Purvis & Hansson, 1981). Changes in enzymatic activities: such as esterase (Molenaar, Rommerts & van der Molen, 1986), 5α-reductase (van der Molen & Rommerts, 1981) and aromatase (Rommerts, de Jong, Brinkmann & van der Molen, 1982) have also been reported. During this differentiation process the interstitial tissue may contain precursors for Leydig cells as well as Leydig cells at different stages of development. For example, the cholesterol side chain cleavage enzyme (CSCC) activity may be active in some cells before they can be regulated by LH, or cells may respond to LH before the appearance of the endoplasmic reticular enzymes, required for the production of androgens. If this occurs the interstitial cell preparation isolated from immature rats may be heterogeneous in functional properties, which may be a disadvantage of this cell preparation for the investigation of the mechanism of regulation of steroid production.

We have characterized with morphological and biochemical techniques the interstitial cell preparation isolated from immature rats, and the results were compared with results of preparations isolated from mature rats.

Particular attention was given to the presence of contaminating macrophages, because these cells may contribute to the steroid production of Leydig cells (Milewich, Chen, Lyons, Tucker, Uhr & MacDonald, 1982; Milewich, Lipscomb, Whisenant & MacDonald, 1983; Lofthus, Marthinsen & Eik-Nes, 1984).

MATERIALS AND METHODS

Ovine LH (NIH-LH0S18); 1.03 IU/mg) was a gift from the Endocrinological Study Section of the National Institute of Health, Bethesda, MD, USA.

Isolated cells were incubated in modified Eagle's medium with Earle's salts and non-essential amino acids containing 100 µg streptomycin/ml, 0.6 µg fungizone/ml and 1% (v/v) fetal calf serum (Gibco, Grand Island, NY, USA). Trypan blue (Gibco), 25-hydroxycholesterol (5-cholesten-3β,25-diol, Steraloids, Wilton, NH, USA), 22R-hydroxycholesterol (5-cholesten-3β,22[R]-diol), dibutyryl cyclic AMP (dbcAMP) (Sigma, St Louis, MO, USA) were used at the indicated concentrations.

Mature (60-90 days old) or immature (21-24 days old) rats from a Wistar substrain (R-Amsterdam) were used. Leydig cells were isolated through collagenase treatment of decapsulated testes as described previously (Rommerts et al., 1985). In the procedure for isolation of Leydig cells from mature rats a Ficoll purification step was routinely employed (Rommerts et al., 1985). Subsequently, the cells were allowed to attach to Costar multiwells or Lux dishes. After 1 h the floating cells were removed by washing, and the cells attached to the dishes were used for the experiments. In the case of the mature rat Leydig cells, the number of attached cells was calculated as described by Molenaar et al. (1983). More than 90% of the interstitial cell preparation isolated from immature rats attached to the culture well and no correction was applied. Incubations were carried out at 32 C (5% CO_2 in air) in the presence of inhibitors of pregnenolone metabolism (cyanoketone, 2α -cyano-17β -hydroxy-4,4',17α -trimethyl-5-androsten-3-one; 5 µmol/l) and SU-10603 (7-chloro-3,4-dihydro-2(3-pyridyl)-1-(2H)-naphtalen-one; 19 µmol/l) (van der Vusse, Kalkman & van der Molen, 1974). At the end of the incubation the medium was collected for estimation of pregnenolone by radioimmunoassay (van der Vusse, Kalkman & van der Molen, 1975). The smallest amount that could be detected was 5 to 10 pmol pregnenolone, the intra-assay variation was in the order of 20%, and the recovery of added pregnenolone was 107% ± 11%.

For the Percoll density gradient centrifugation experiments interstitial cells from immature rats (3-6 animals) were taken up in 1 ml 0.15 M NaCl, 0.1% (w/v) BSA containing 1 mg/ml DNase (Sigma, St Louis, MO, USA). The cells were layered on a pre-formed continuous 30-60% Percoll gradient in 0.15 M NaCl, 0.1% (w/v) BSA and centrifuged for 20 minutes at 1500xg.

The gradient was fractionated by puncturing the bottom of the tube, and 0.5 ml fractions were collected by pumping air in the top of the tube. Small aliquots were counted and assayed histochemically for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity (Rommerts & Molenaar, 1985). Each fraction was divided into equal parts in two culture dishes for determination of steroid production.

For labelling of macrophages, animals were injected i.p. with 3% (w/v) trypan blue in saline (10 ml/kg body weight) 2-3 days before they were killed. For morphology, testes were fixed by perfusion fixation with 2% (v/v) glutaraldehyde, 3% (v/v) formaldehyde, 3% (w/v) polyvinylpyrrolidon in 0.1 M phosphate buffer pH 7.3 (Forssmann, Ito, Weine, Aoki, Dym & Fawcett, 1977). Isolated cells, attached to plastic, were fixed in the same fixative after a one hour attachment period. Testes and cells were embedded in Epon and 1 µm sections were cut and stained with toluidine blue.

RESULTS

A. Morphological characterization of the interstitial cell preparations from immature and mature rats

In the isolated cell preparations, Leydig cells were identified by staining for 3β-HSD activity in vitro, and macrophages by the presence of phagocytized trypan blue (injected in vivo), respectively. The results in

Table 1. Proportions of Leydig cells and macrophages in interstitial cell preparations isolated from mature and immature rats.

Preparation	Leydig cells (%)	Macrophages (%)
immature	43 ± 7	-
mature	80 ± 7	20 ± 6

Leydig cell number was determined by staining for 3β-hydroxysteroid dehydrogenase activity. Macrophages were identified by phagocytosis of trypan blue injected in vivo three days before killing the animals. Values given are means ± SD of 5 different cell preparations of each age.

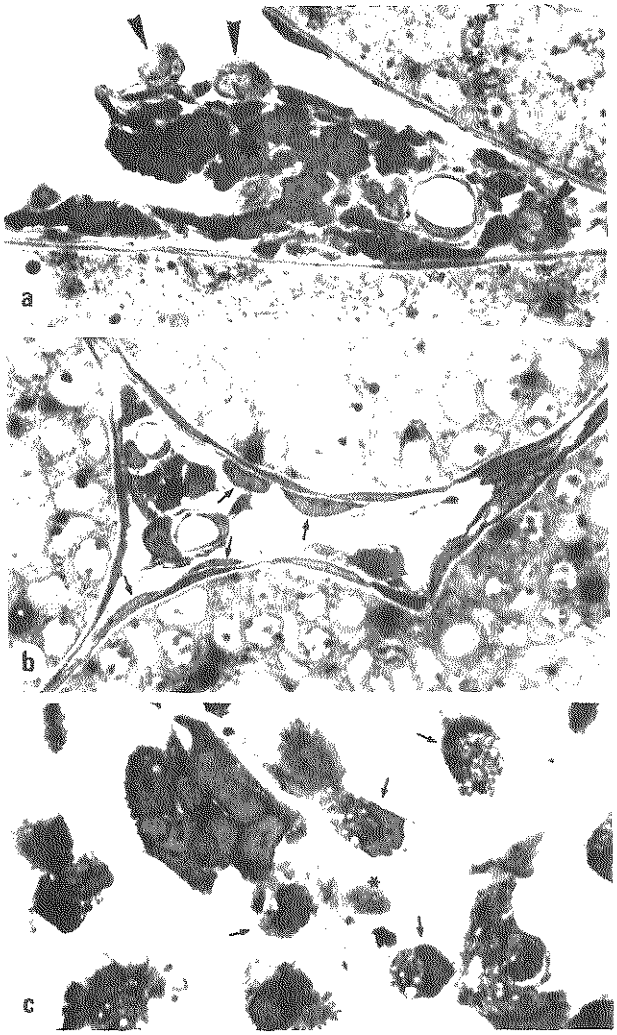

Figure 1
a. Cross-section of a mature rat testis (x720)
✱: cluster of Leydig cells
➤: macrophages
b. Cross-section of an immature rat testis (x720)
✱: cluster of Leydig cells
→: peritubular cells
c. Section of an interstitial cell preparation from immature rats (x720)
✱: cluster of Leydig cells
→: mesenchymal cells
∗: undefined cells
Testes (a,b) and cells (c) were fixed, and embedded as described in the Materials and Methods section. 1 µm sections were cut and stained with toluidine blue.

table 1 show that the Ficoll purified preparation from mature animals contained approximately 80% Leydig cells and 20% macrophages, whereas preparations from immature rats, without application of cell purification techniques contained only 43% Leydig cells and virtually no macrophages.

We have considered the possibility that the preparation from immature rats contained macrophages not active in phagocytosis, and in this regard we have characterized the preparations by also determining the structural morphological properties of the cells. Macrophages which can be characterized by a pale staining cytoplasm containing large vacuoles, and an irregular pale staining nucleus with clumped peripheral heterochromatin (Laws, Wreford & de Kretser, 1985; Kerr & Sharpe, 1985), were frequently seen in the interstitial tissue from mature rats (fig. 1A), whereas these cells were very rare in the interstitial tissue from immature rats (fig. 1B). Leydig cells characterized by a round or oval dark staining nucleus, containing prominent nucleoli and a thin rim of peripheral heterochromatin, and a dark staining cytoplasm (Laws et al., 1985; Kerr & Sharpe, 1985) were clearly present in both preparations. Virtually no macrophages were seen in the cell preparations isolated from immature cells (fig. 1C). Two different cell types were predominant in this preparation: Leydig cells, and cells containing a crescent-shaped eccentric nucleus and many interdigitating processes, which we have called mesenchymal cells (table 2). We have considered the possibility that the latter fraction contained endothelial cells. Staining for alkaline phosphatase and binding of acetyl-LDL, both characteristics of endothelial cells (Voyta, Via, Butterfield & Zetter, 1984), showed less than 2% positive cells, indicating the very low number of endothelial cells in the preparation (results not shown). The proportion of Leydig cells based on morphological criteria (table 2) was significantly higher than the figure based on 3β-HSD histochemistry ($P<0.01$; Student's t-test).

B. Percoll density gradient purified cells.

To investigate the possible presence of Leydig cells in different stages of development in the interstitial cell preparation from immature rats, a 30 - 60% Percoll density gradient centrifugation method was employed. After fractionation of the gradient the steroidogenic properties of the cells were determined by measuring the LH-stimulated pregnenolone

Table 2. Morphological determination of the composition of the interstitial cell preparation from immature rats

Cell type	Percentage of total
Leydig cells	58 ± 2
Mesenchymal cells	37 ± 4
Undefined cells	7 ± 4

Cells were characterized according to the criteria described in the text. Results are given as means ± SD of three different cell preparations. The percentage Leydig cells.

production, and the pregnenolone production in the presence of 25-hydroxycholesterol (30 µmol/l) (fig. 2A). The presence of 3β-HSD activity was determined with histochemistry, performed on a small aliquot of each fraction (fig. 2B). Fraction 1 (from the bottom of the tube) contained mostly erythrocytes, whereas the germinal cells concentrated in fractions 14-17. Fractions 10-12 of the gradient contained a large number of small Sertoli cell aggregates to which Leydig cells may have adhered. This may account for the aberrant results obtained with these fractions.

The highest LH- as well as 25-hydroxycholesterol-stimulated pregnenolone productions were found in fraction 7. This fraction also contained the highest number of 3β-HSD-positive cells. The distribution of the LH- and 25-hydroxycholesterol-stimulated pregnenolone and the 3β-HSD-positive cells through the gradient is similar. The LH-stimulated pregnenolone production when calculated per 10^6 3β-HSD-positive cells is between 30 and 135 pmol/h (100-430 pmol/h for the 25-hydroxycholesterol-dependent production). The range in these figures is probably the result of the inaccuracy of the estimation of the percentage of 3β-HSD-positive cells. The pregnenolone productions expressed per 10^6 3β-HSD-positive cells appeared not to be different in the various fractions, indicating that the steroid production by these cells is not affected by the 3β-HSD-negative cells present in the preparation. A preparation containing 90% 3β-HSD-positive cells can be obtained by pooling fractions 2-8 of the gradient. The total yield with this method is approximately 25%.

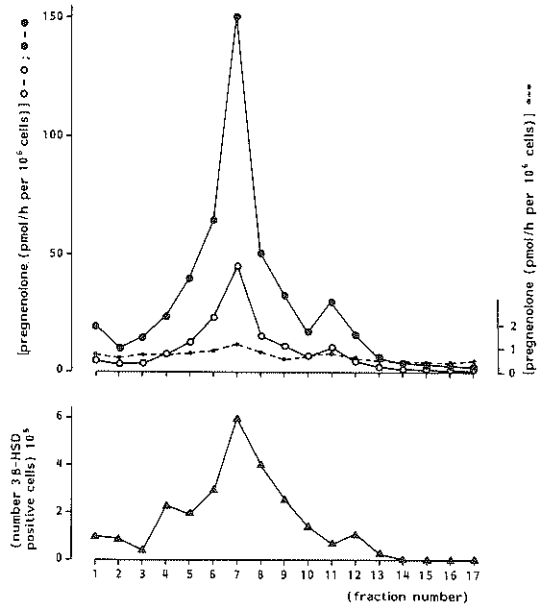

Figure 2
Top: LH- and 25-hydroxycholesterol-dependent pregnenolone productions by interstitial cells from immature rats after separation on a 30-60% Percoll density gradient. Fraction 1 was the most dense fraction. The pregnenolone production is expressed per total cell number. The results from one typical experiment out of three are shown: (.--.: controls; o-o: 100 ng/ml LH; ●-●: 30 µmol/l 25-hydroxycholesterol).
Bottom: Total number of 3β-HSD-positive cells present in the fractions.

C. Steroidogenic activities of Leydig cells from mature and immature rats.

Pregnenolone productions by interstitial cells isolated from mature and immature animals were compared under basal conditions, after stimulation with LH (100 ng/ml) or dbcAMP (0.5 mmol/l), or in the presence of 22R-hydroxycholesterol (19 µmol/l) (fig. 3). The steroid production calculated per 3β-HSD positive cell by cells from mature rats was under all incubation conditions at least four times higher than the production under corresponding conditions by Leydig cells from immature rats.

We have also considered the possibility that the 20% macrophages present in the interstitial cell preparation from mature rats could have contributed to the level of pregnenolone production. However, in Leydig

cell-free, macrophage-enriched cell preparations (obtained after in vivo administration of ethane dimethylsulfonate, which is cytotoxic for Leydig cells; Molenaar et al., 1986), no pregnenolone production could be found after stimulation of the cells with LH or 22R-hydroxycholesterol (results not shown).

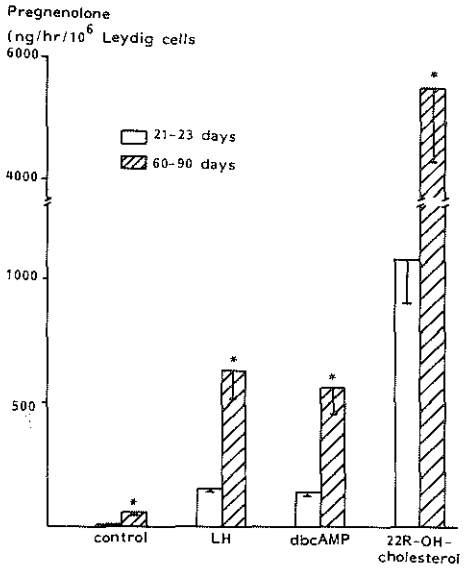

Figure 3. LH-, dbcAMP- and 22R-hydroxycholesterol-dependent pregnenolone productions by interstitial cell preparations obtained from immature (open bars) and mature (hatched bars) rats. The following concentrations were used: LH: 100 ng/ml; dbcAMP: 0.5 mmol/l; 22R-hydroxycholesterol: 19 µmol/l. The pregnenolone production is expressed per 3β-HSD-positive cell. The Leydig cell preparations from immature and mature rats contained 43 ± 7 and 80 ± 7% 3β-HSD-positive cells respectively. Means ± SD of 5 different cell preparations are given.
*Significantly different ($P<0.01$) from preparation isolated from immature rats (Student's t-test).

DISCUSSION

Leydig cells from immature rats have been used frequently for the investigation of hormonal regulation of steroid production (Bambino, Schreiber & Hsueh, 1980; Meidan, Lim, McAllister & Hsueh, 1985; Tsai-Morris, Aquilano & Dufau, 1985; Themmen et al., 1985, 1986). The interstitial cell preparation from the immature rat has some advantages: the cells can be obtained relatively easy with a high yield and viability, and can be

cultured for at least 72 h without loss of LH-dependent steroid production (Molenaar et al., 1983; Rommerts & Molenaar, 1985; Berns et al., 1985). However, the preparation is not pure, because at this age the Leydig cell population undergoes developmental changes, and the interstitial cell preparations may contain Leydig cells that are in different stages of development (Knorr et al., 1970; Lording & de Kretser, 1972; Tapanainen et al., 1984).

In the mature rat, the yield of Leydig cells is low, thus the final cell preparation may not represent the original Leydig cell population in the testis. We have also found that the preparation still contains about 20% macrophages (table 1). Several steroidogenic activities have been reported for macrophages, e.g. 17α-hydroxylase in adrenal macrophages (Vernon-Roberts, 1969), and 3β-HSD, 3α-HSD, 17β-HSD and 5α-reductase were observed in peritoneal and alveolar macrophages (Milewich et al., 1982, 1983; Lofthus et al., 1984). However, no pregnenolone production could be found after stimulation of testicular macrophages isolated from mature rat testes. In cell preparations from immature rats no macrophages could be detected either by phagocytic properties or by morphological criteria. In these preparations approximately 37% of the cells present were very similar to the mesenchymal cells described in testis sections by Kerr & Sharpe (1985) and in isolated cell preparations by Laws et al. (1985). Therefore we have referred to this fraction as mesenchymal cells. It is generally accepted that the adult population of Leydig cells arises from these mesenchymal cells (Hooker, 1970; Christensen, 1975), and it may be possible that these cells have some of the characteristic properties of Leydig cells. We have separated therefore the isolated interstitial cells from immature rats using density gradient centrifugation. The steroidogenic activity of the different fractions was estimated. 3β-HSD activity was used as a marker for microsomal enzymes, the cholesterol side chain cleavage enzyme activity was measured as a mitochondrial enzyme marker using 25-hydroxycholesterol (Mason & Robidoux, 1978; Brinkmann, Leemborg, Rommerts & van der Molen, 1984) and the coupling of the mechanism involved in the hormonal regulation of steroidogenesis was determined by measuring the LH-dependent steroid production. It was shown that all these different activities are distributed in a similar fashion through all the fractions of the gradient. This may indicate that development of the LH-dependent steroidogenic apparatus occurs coordinated with similar kinetics. These results also show

that the 3β-HSD-negative cells are probably not steroidogenic, and have no major effect on the steroid production in Leydig cells. Hence, the interstitial cell preparation isolated from immature rat testis is suitable for studies on the regulation of steroid production. The discrepancy between the percentage Leydig cells based on morphological criteria and 3β -HSD activity may indicate that Leydig cells acquire their morphological characteristics prior to their steroidogenic activities.

Purvis, Clausen & Hansson (1978) and van der Molen, Roodnat & Rommerts (1980) have reported that the steroidogenic capacity of the Leydig cell in the developing testis is constant. We have demonstrated in earlier studies (Molenaar et al., 1983) that Leydig cell preparations obtained from mature rats contain damaged cells and that selection of intact cells resulted in a significant increase in testosterone production per cell. Hence it seems likely that in the studies mentioned above the steroidogenic capacity of Leydig cells from mature rats has been underestimated. In the present study we have shown that the 3β-HSD-negative cells do not contribute to steroid production, and that 3β-HSD activity may be used as a marker for steroidogenically active Leydig cells. The results in figure 3 show that Leydig cells from mature rats have a fourfold larger capacity for production of pregnenolone compared to cells obtained from immature rats.

The stimulatory effect of dbcAMP was not different from the effect of LH indicating that the lower number of LH receptors in Leydig cells from immature rats is not responsible for the difference in steroid production. Since pregnenolone production in the presence of 22R-hydroxycholesterol reflects the cholesterol side chain cleavage activity, the capacity of this enzyme also increases during maturation.

In conclusion, we have shown that interstitial cell preparations isolated from immature rats contain 40-60% Leydig cells (depending on the chosen parameter), cells of mesenchymal origin and virtually no macrophages. All cells that show 3β-HSD activity are also LH-responsive and have CSCC activity, suggesting that the different components of the steroidogenic apparatus develop simultaneously in the Leydig cell. The steroidogenic capacity of the Leydig cell appears to increase fourfold during testicular development.

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APPENDIX PAPER 4

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The possible role of protein kinase C and phospholipids in the regulation of steroid production in rat Leydig cells

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We have studied the possible involvement of the activation of calcium-dependent phospholipid-activated protein kinase (PK-C) in the stimulatory action of LHRH on Leydig cells, using 4β-phorbol-12-myristate-13-acetate (PMA) and phospholipase C (PL-C). LHRH agonist (LHRH-A) and PL-C had a large synergistic effect on LH-stimulated steroid production, whereas PMA inhibited the effect of LH. However, PMA always caused an increase in steroid production stimulated by various doses of dibutyryl cAMP. LH and PMA stimulated the phosphorylation of 17 and 33 kDa proteins, whereas LHRH-A and PL-C had no effect. Of all effectors used, LH had the most pronounced effect on the synthesis of 14, 27 and 30 kDa proteins. The present results suggest that the mechanisms of action of LHRH-A and PL-C on steroid production in Leydig cells may be similar and different from PMA, and may involve stimulation of a specific type of PK-C or hydrolysis of a specific pool of phospholipids.

(Leydig cell) Phorbol ester Phospholipase C Steroidogenesis LH LHRH

1. INTRODUCTION

It is generally accepted that cAMP plays an important role in the regulation of steroid production in testicular Leydig cells, since stimulation of the mitochondrial side chain cleavage enzyme by LH is accompanied by increased levels of cAMP, activation of protein kinase A and phosphorylation of at least six proteins [1–3]. Recently it was shown that LH action occurs concomitant with changes in the intracellular concentration of Ca^{2+} [4] and an increase in PI metabolism [5].

The pituitary decapeptide luteinizing hormone releasing hormone and its analogues (LHRH-A) have direct effects on Leydig cell steroid production [6,7], but the mechanism of action of LHRH or LHRH-A on Leydig cell steroidogenesis is not clear. Results from different investigators indicate that the action of LHRH is not mediated by cAMP [8,9]. It has been shown with isolated Leydig cells from mature rats that LHRH-A can cause an increase in the intracellular Ca^{2+} level [4] and a stimulation of incorporation of labelled phosphate into PI [10]. These results indicate that the action of both LH and LHRH involves changes in intracellular Ca^{2+} and PI turnover, although these hormones have different effects on cAMP and steroid production. Increased PI turnover may lead to production of diacylglycerol with subsequent stimulation of calcium-dependent phospholipid-activated protein kinase (PK-C) [11].

To investigate to which degree PK-C activation may play a role in the regulation of steroid production by LHRH, we have compared the steroidogenic effects of PMA and PL-C with those of LHRH-A. In addition, we have measured the effects of these agents on cellular protein phosphorylation and protein synthesis which may

Abbreviations: dbcAMP, N^6-2′-*O*-dibutyryl adenosine cyclic 3′,5′-monophosphate; PL-C, phospholipase C; PL-A_2, phospholipase A_2; PMA, 4β-phorbol-12-myristate-13-acetate; PA, 4β-phorbol-13-monoacetate; PI, phosphatidylinositol

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reflect cellular activities after stimulation of specific membrane transducing systems.

2. MATERIALS AND METHODS

2.1. *Materials*

PMA, PA and PL-C (*Clostridium perfringens*, type XII) were obtained from Sigma (St. Louis, MO). Ovine LH (NIH-LH-S18, 1.03 IU/mg) was a gift from the Endocrinological Study Section of the National Institute of Health, Bethesda, MD. (D-Ser-t-bu^6,des-Gly^{10}-NH_2)-LHRH-ethylamide (HOE766, LHRH agonist) and Ac-(D)-pC1Phe-(D)pC1Phe-(D)Trp-Ser-Tyr-(D)Phe-Leu-Arg-Pro-(D)Ala-NH_2 (ORG30093, an LHRH antagonist) were kindly provided by Hoechst Pharma (Amsterdam, The Netherlands) and Organon (Oss, The Netherlands) respectively.

2.2. *Methods*

Leydig cells were isolated from 21–24-day-old rats and incubated as described [9,12]. Pregnenolone was measured by radioimmunoassay in the medium after incubation of the cells for 3 h with the indicated additions in the presence of inhibitors of pregnenolone metabolism cyanoketone (5 μM) and SU-10603 (19 μM) [13,14].

Protein phosphorylation was measured in intact cells. Cells (10^6) were incubated for 3 h in 0.5 ml Krebs-Ringer buffer without phosphate but containing 0.2% (w/v) glucose, 0.1% (w/v) BSA, in the presence of 100 μCi $^{32}PO_4$ (carrier free). The proteins were extracted and separated using polyacrylamide gradient (8–15%) gel electrophoresis in the presence of SDS as described by Bakker et al. [15]. For protein synthesis 10^6 cells were incubated in medium with 15–30 μCi [^{35}S]methionine. Cells were sonicated (2 times for 5 s, high setting) in 1 ml of 10 mM Tris-HCl (pH 7.4), 0.16 M NaCl, 3 mM $MgCl_2$, 5 mM KCl, 1 mM PMSF, and centrifuged (25 min, 10000 × *g*). Proteins were precipitated from the supernatant with 10% trichloroacetic acid, and separated on polyacrylamide gradient (10–20%) gels in the presence of SDS. The gels were dried and autoradiography was performed using Kodak SB-5 X-ray film. M_r was determined using an electrophoresis calibration kit from Pharmacia (Uppsala).

3. RESULTS

Isolated Leydig cells from immature rats were incubated with different amounts of LH in the absence or presence of 40 nM LHRH-A (fig.1A).

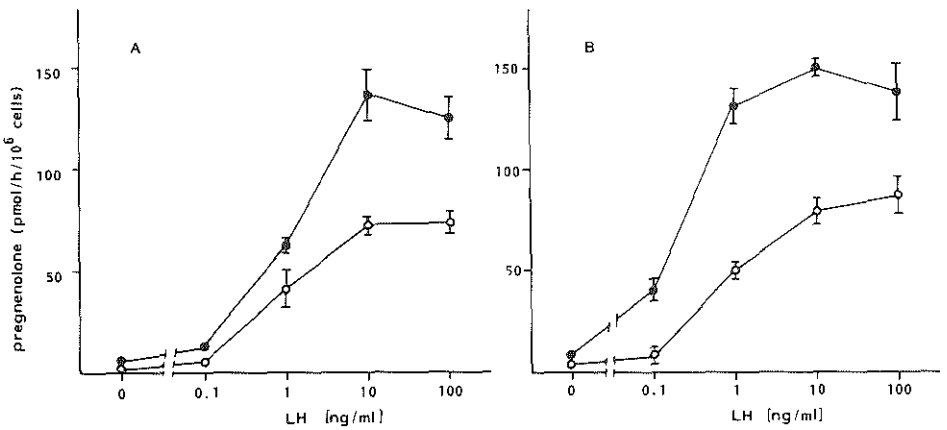

Fig.1. (A) Effect of LHRH-A on LH-stimulated pregnenolone production. Leydig cells were incubated with different amounts of LH for 3 h in the absence (○) or presence (●) of 40 nM LHRH-A. (B) Effect of PL-C on LH-stimulated pregnenolone production. Leydig cells were incubated with different amounts of LH for 3 h in the absence (○) or presence (●) of 1 U/ml PL-C. The values given are means ± SD (n = 4) of two different cell preparations. Steroid productions in the presence of LHRH-A or PL-C are significantly ($P < 0.01$) different from their respective controls (Student's t-test).

Pregnenolone production was stimulated up to 35-fold by LH alone, and could be further stimulated (60-fold) by the addition of 40 nM LHRH-A. LHRH-A alone also stimulated pregnenolone production in a dose-dependent manner with an ED_{50} of 0.1 nM LHRH-A and a 5-fold maximal stimulation (basal: 2.0 ± 0.1; 40 nM LHRH-A: 9.6 ± 0.2 pmol pregnenolone/h per 10^6 cells). All effects of LHRH-A on steroid production were completely abolished when the LHRH antagonist ORG30093D (400 nM) was included in the incubation medium (not shown).

To investigate the effect of activation of PK-C on steroid production we incubated cells with increasing concentrations of LH in the presence of the phorbol ester PMA (100 ng/ml) (fig.2A). PMA alone stimulated steroid production 3-fold, but inhibited steroidogenesis at intermediate concentrations of LH, without affecting maximally stimulated cells. Results from incubation of cells with PMA in the presence of different concentrations of dbcAMP showed that the small steroidogenic effect of PMA was constant.

PL-C (1 U/ml) was added to the incubations to study the effects of phospholipid hydrolysis on Leydig cell steroid production (fig.1B). PL-C stimulated basal pregnenolone production 3-fold, and was able to stimulate LH-dependent steroid production further. These effects of PL-C were similar to those of LHRH-A.

To characterize further the effects of LH, LHRH-A, PL-C and PMA we have analyzed protein phosphorylation and protein synthesis by Leydig cells. During a 3 h incubation, LH as well as PMA stimulated the phosphorylation of a 17 and a 33 kDa protein, whereas addition of LHRH-A or PL-C had no discernable effects on protein phosphorylation. LH, LHRH-A, PL-C and PMA all stimulated the synthesis of 14, 27 and 30 kDa proteins after 5 h incubation, but the effects of LHRH-A, PL-C and PMA were much less pronounced than the effects of LH.

Addition of the phorbol ester PA (100 ng/ml) or $PL\text{-}A_2$ (5 U/ml) did not have any effects on steroid production, protein phosphorylation or protein synthesis.

4. DISCUSSION

The effects of two activators of PK-C on signal transduction pathways for regulation of steroid production in Leydig cells have been investigated: PMA which has been shown to activate PK-C [16] and PL-C which increases the concentration of

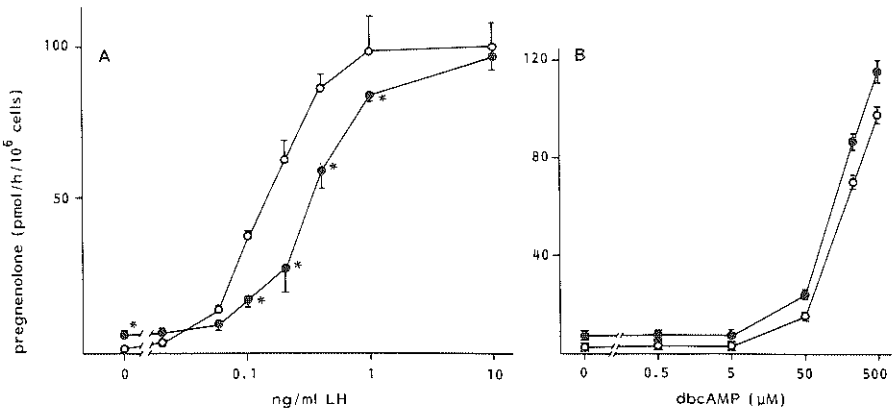

Fig.2. (A) Effect of PMA on LH-stimulated pregnenolone production. Leydig cells were incubated with different amounts of LH for 3 h in the absence (○) or presence (●) or 100 ng/ml PMA. (B) Effect of PMA on dbcAMP-stimulated pregnenolone production. Leydig cells were incubated with different amounts of dbcAMP in the absence (○) or presence (●) of 100 ng/ml PMA. The values given are means ± SD ($n = 4$) of two different cell preparations. Steroid productions in the presence of LHRH-A or PL-C are significantly ($P < 0.01$) different from their respective controls (Student's t-test).

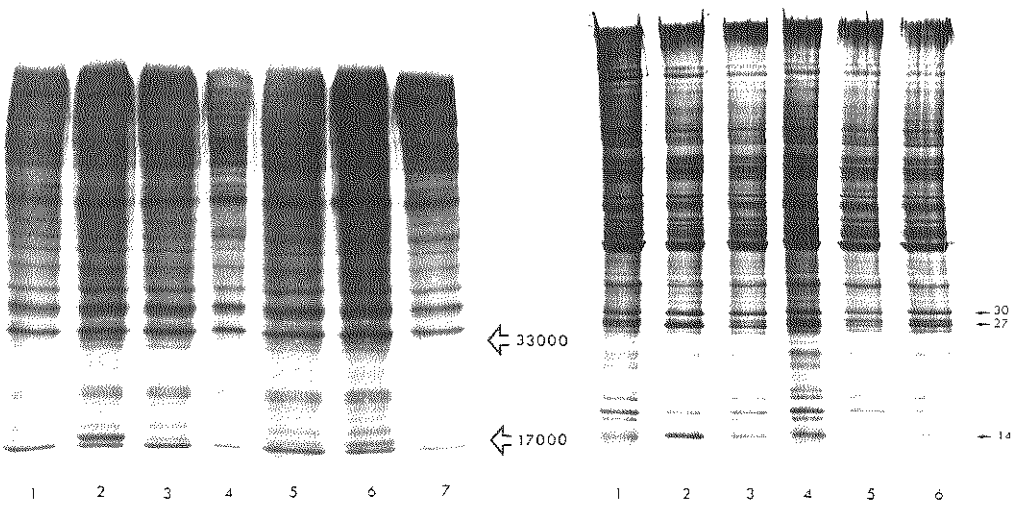

Fig.3. (Left) Protein phosphorylation patterns of Leydig cells. Cells were incubated for 3 h in the presence of $^{32}PO_4$. Proteins were extracted and separated on SDS-PAGE. The autoradiograph of the dried gel is shown. Lanes: 1, control; 2, 100 ng/ml LH; 3, 40 nM LHRH-A; 4, 1 U/ml PL-C; 5, 5 U/ml PL-A_2; 6, 100 ng/ml PMA; 7, 100 ng/ml PA. (Right) Protein synthesis pattern of Leydig cells. Cells were incubated for 5 h in the presence of [^{35}S]methionine. Proteins were extracted and separated on SDS-PAGE. The autoradiograph of the dried gel is shown. Lanes: 1, control; 2, 100 ng/ml LH; 3, 40 nM LHRH-A; 4, 100 ng/ml PMA; 5, 100 ng/ml PA; 6, 1 U/ml PL-C.

diacylglycerol in membranes [17,18] and elicits effects on protein phosphorylation in 3T3 cells which are supposed to be mediated by PK-C [19].

The synergistic effect of LHRH-A on LH-dependent steroid production was not observed with PMA. The phorbol ester stimulated basal steroid production and slightly inhibited the LH effects, but no inhibitory effects on dbcAMP- or maximally LH-stimulated steroidogenesis were found. These results indicate that PMA had no deleterious effects on the cells. Similar results have been obtained for mouse Leydig cells, and the inhibitory effect of PMA appeared to be located at the level of the regulatory GTP-binding protein of adenylate cyclase [20].

PL-C and LHRH-A stimulated basal steroid production as well as LH-dependent steroidogenesis, suggesting a common mechanism of action of PL-C and LHRH-A. LHRH-A and PL-C had also similar effects on protein phosphorylation and protein synthesis: i.e. the rate of protein phosphorylation was not affected, but the synthesis of proteins of 14, 27 and 30 kDa was slightly increased. These results suggest that LHRH and PL-C may share a common mechanism of action. In contrast, PMA stimulated protein phosphorylation to approximately the same extent as LH, but the effect of PMA on the newly synthesized proteins was much less pronounced than the effect of LH. These results indicate that patterns of protein synthesis are more sensitive to detecting actions of extracellular stimuli than protein phosphorylation patterns. Enzymatic amplification steps following protein phosphorylation could contribute to this.

PL-C has been used also in other studies on regulation of steroidogenesis. PL-C had a strong synergistic effect on ACTH-stimulated cortisol production in isolated bovine adrenocortical cells [17], but inhibitory effects on Leydig cells have been reported also [21]. An explanation for these different effects of PL-C may be that these authors used a PL-C from another microbial source (*Bacillus cereus*). Since it has been shown in murine epidermal cells and in GH_4C_1 pituitary cells, that PL-C from *B. cereus* had very small effects compared to PL-C from *C. perfringens*, the

enzymes may have a different substrate specificity [18,22]. These results indicate that specific phospholipids in the cell membrane may be involved in the control of specific protein kinases. Similar specific phospholipids may be hydrolyzed after activation of the cells by LHRH-A or PL-C resulting in activation of a different type of protein kinase, which is not activated by PMA [23]. Alternatively, PL-C may cause changes in membrane fluidity, which have been shown to influence the number of LH receptors and their coupling to the adenylate cyclase system [24]. Arachidonic acid metabolites, which have been implicated to play a role in the regulation of steroid production [4], are not likely to be involved because phospholipase A_2 did not have any effect.

In conclusion, the present results show that the mechanisms of action of PMA and PL-C on Leydig cell steroidogenesis are clearly different. The similarities between the effects of LHRH-A and PL-C suggest that one primary effect of LHRH on Leydig cells may be the stimulation of hydrolysis of specific phospholipids. It appears therefore important to elucidate further the exact molecular mechanism of action of PL-C on these cells.

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APPENDIX CHAPTER

STIMULATORY AND INHIBITORY GUANINE NUCLEOTIDE BINDING PROTEINS ARE PRESENT IN RAT LEYDIG CELLS

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Abbreviations: LH: luteinizing hormone; LHRH-(A): luteinizing hormone-releasing hormone (agonist); GTPγS: guanosine-5'-(3-O-thio)-trisphophate; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA: bovine serum albumin

ABSTRACT

Incubation of a crude membrane preparation of rat Leydig cells with cholera toxin in the presence of GTP and (^{32}P)-NAD resulted in a toxin dependent (^{32}P)-ADP-ribosylation of a 44 kDa protein, which is probably the α-subunit of the stimulatory guanine nucleotide binding protein (N_s). The functional relevance of N_s was indicated by the ability of cholera toxin to stimulate basal and LHRH-A-dependent pregnenolone production. The presence of an inhibitory guanine nucleotide binding protein (N_i) was investigated by incubating Leydig cell membranes in the presence of GDP and (^{32}P)-NAD. Pertussis toxin stimulated the labelling of a 39 kDa protein, which is probably the α-subunit of N_i. Pertussis toxin treatment of the cells for 2 h did not change the LH- and LHRH-A-dependent pregnenolone production in Leydig cells. However, when cells were incubated for 24 h with pertussis toxin, the basal, LH- and cholera toxin-dependent pregnenolone production was stimulated. We conclude from these results that both N_s and N_i are involved in the regulation of steroidogenesis in rat Leydig cells. N_s is probably activated by LH and activates adenylate cyclase, whereas the role of N_i in the regulation of Leydig cell steroidogenesis remains to be elucidated.

INTRODUCTION

Steroid production in rat Leydig cells is mainly under the control of the pituitary hormone LH. Stimulation of the mitochondrial cholesterol side chain cleavage enzyme (CSCC) by LH is accompanied by increased levels of cAMP, activation of cAMP-dependent protein kinase and increased phosphorylation of at least six proteins (Cooke et al, 1976; Podesta et al, 1978; Bakker et al, 1983). The pituitary decapeptide LHRH can stimulate also steroid production in Leydig cells. However, the mechanism of action is not clear. Both LH and LHRH-agonist (LHRH-A) stimulate intracellular calcium levels (Sullivan & Cooke, 1986).

Adenylate cyclase activity can be regulated in many different cells by stimulatory and inhibitory signals (Gilman, 1984; Birnbaumer et al, 1985). The effects of stimulatory agonists are mediated by a stimulatory guanine nucleotide binding protein that stimulates adenylate cyclase activity (N_s), whereas the inhibitory signals are transduced by an analogous, but

different inhibitory guanine nucleotide binding protein (N_i). It has been observed that the activation of adenylate cyclase in Leydig cell membrane preparations by hCG is dependent on the presence of guanine nucleotides, suggesting that the effect of LH might be mediated by N_s (Dufau et al, 1980; Mukhopadhyay & Schumacher, 1985).

An inhibitory protein that modulates adenylate cyclase activity (N_i) has not yet been demonstrated in Leydig cells, although the presence of N_i could have major implications in the regulation of Leydig cell functions. N_i may mediate effects of inhibitory agonists on Leydig cells, such as arginine vasotocin (Adashi et al, 1984) or LHRH, which has been shown to inhibit LH-stimulation of cAMP levels (Sullivan & Cooke, 1984). Furthermore, there are indications that N_i is involved in the regulation of calcium channels in cells (Koch et al, 1985; Okajima et al, 1985; Holz et al, 1986). It has been shown that LH and LHRH-A stimulate intracellular calcium levels in Leydig cels. (Sullivan & Cooke, 1986), and N_i may be involved in this action of LH and LHRH.

For these reasons we have investigated whether N_s and N_i are present in mature rat Leydig cells using the ability of cholera toxin and pertussis toxin to stimulate the ADP-ribosylation of the α-subunits of N_s and N_i, respectively (Cill & Meren, 1977; Katada et al, 1982; Gilman, 1984). In addition to these efforts to demonstrate N_s and N_i, we also have incubated intact Leydig cells with the toxins to examine the functional role of N_s and N_i in the regulation of steroid production in the Leydig cell.

MATERIALS AND METHODS

Pertussis toxin (Kakeu Pharmaceutical Co., Tokio, Japan) and cholera toxin (List Biological Laboratories, Campbell, CA, U.S.A.) were used at the indicated concentrations. Ovine LH (NIH-LH-S18; 1.03 i.u./mg) was a gift from the Endocrinology Study Section of the National Institutes of Health, Bethesda, MD, U.S.A. (D-ser(t-bu)^6,des-gly^{10}-NH_2)LHRH-ethylamide (HOE 766, an LHRH-agonist) was a kind gift from Hoechst Pharma (Amsterdam, The Netherlands). (^{32}P)-NAD was prepared as described by Cassel & Pfeuffer (1978).

Ficoll-purified Leydig cells were isolated from mature (6-9 weeks old) rats as described previously (Rommerts et al, 1985). Cells were incubated in Costar 96 multiwell dishes in 200 μl modified Eagle's medium with

Earle's salts and non-essential amino acids containing 100 μg streptomycin/ml, 0.6 μg fungizone/ml, 100 i.u. penicillin/ml and 1% (v/v) fetal calf serum (Gibco, Grand Island, NY, U.S.A.). After 1 h the floating cells were removed by washing, and the cells attached to the dishes were used for the experiments. Incubations were carried out at 32°C (5% CO_2 in air) in the presence of the pregnenolone metabolism inhibitors cyanoketone (5 μmol/l) and SU-10603 (19 μmol/l) (van der Vusse, Kalkman, & van der Molen, 1974). At the end of the incubation the medium was collected for determination of pregnenolone by radioimmunoassay (van der Vusse, Kalkman & van der Molen, 1975). The smallest amount that could be measured was 5 to 10 pmol pregnenolone, the inter-assay variation was in the order of 20%, and the recovery of added pregnenolone was 107%±11%.

For the ADP-ribosylation experiments a crude membrane preparation of mature rat Leydig cells was prepared. Attached cells were harvested using a Costar cell scraper in 10 mM Tris-HCl pH 7.4, 0.16 M NaCl, 3 mM $MgCl_2$, 5 mM KCl (TBSS). The cells were washed in TBSS by centrifugation (200xg, 2 min) and the pellet was resuspended in tenfold diluted TBSS. After 5 minutes the cells were broken in a Dounce glass-glass homogenizer (clearance 0.025-0.03 mm; ten strokes), and 0.1 volumes of tenfold concentrated TBSS was added to restore isotonicity. The broken cell suspension was centrifuged for 2 min at 1000xg to remove nuclei. The supernatant was centrifuged for 30 min at 100,000xg, and the crude membrane pellet was resuspended and stored at -80°C. At the start of the experiments the preparation was washed two times with 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and resuspended to obtain a protein concentration of 1-5 mg/ml.

The ADP-ribosylation experiments were carried out essentially as described by Ribeiro-Neto et al (1985) with some modifications. Cholera toxin and pertussis toxin (0.5 mg/ml) were activated by incubation for 30 min at 37°C in the presence of 20 mM dithiotreitol (DTT), and diluted in 0.1% BSA (carrier protein), 20 mM DTT, 40 mM EDTA, 25 mM Tris-HCl pH 7.5. For the ADP-ribosylation 10-50 μg (in 10 μl) of the membranes were mixed with 10 μl 100 μg/ml cholera toxin (10 μl 10 μg/ml pertussis toxin), and 30 μl 10 mM thymidine, 1 mM ATP, 0.1 mN GTPγS, 10 μM NAD^+, 1.5 mM DTT, 20 mM $MgCl_2$, 300 mM potassium phosphate pH 7.5, 25 mM HEPES/Tris pH 7.5, 1 mM EDTA, 0.02 mg/ml DNase (in the case of pertussis toxin: 30 μl 25 mM Tris-HCl pH 7.5, 10 mM thymidine, 5 mM ATP, 1 mM GDP, 1 mM EDTA, 1 μM NAD^+, 0.02 mg/ml DNase). The labelling was started with the addition of $2-4x10^6$

cpm of (^{32}P)-NAD, and the tubes were incubated for 1 h at 30°C. The incubation was stopped by the addition of 9 volumes of acetone at 0°C. The protein pellet was washed with 10% (v/v) TCA, chloroform, and dissolved in sample buffer (Laemmli, 1970). The proteins were separated with polyacrylamide gel electrophoresis (10%) in the presence of sodiumdodecylsulphate (Laemmli, 1970). The gel was stained and prepared for autoradiography on Kodak SB-5 X-ray film. Molecular weight was determined using an electrophoresis calibration kit from Pharmacia Fine Chemicals (Uppsala, Sweden). The staining of BSA was taken as a qualitative measure of the recovery of the protein samples during the washing procedure.

RESULTS

To investigate the possible presence of N_s and N_i in mature rat Leydig cells, crude Leydig cell membrane preparations were incubated with cholera toxin or pertussis toxin in the presence of (^{32}P)-NAD (Figure 1A,B). Cholera toxin stimulated the labelling of a 44 kDa protein. A smaller protein (28 kDa) was also labelled, but this labelling might reflect the auto-ADP-ribosylation of the 29 kDa A-subunit of cholera toxin (Gill, 1977). Pertussis toxin stimulated the ADP-ribosylation of a 39 kDa protein. In the presence of GTPγS and $MgCl_2$ instead of GDP the labelling of this protein was decreased (not shown). Identical amounts of protein were applied to the gels, as judged by the intensity of the staining of BSA (not shown).

The effect of the toxins on steroid production was also investigated. Leydig cells from mature rats were preincubated for 2 h with 100 ng/ml cholera toxin or pertussis toxin. Subsequently, LH (100 ng/ml) or LHRH agonist (LHRH-A; 40 nM) was added, and the pregnenolone production in the presence of inhibitors of pregnenolone metabolism was estimated during the next 3 h (figure 2). In control incubations LHRH-A and LH both stimulated pregnenolone production (two- and twelvefold, respectively). Preincubation with cholera toxin resulted in a large increase in the control and LHRH-A-dependent steroid production (six- and four-fold respectively), but did not affect LH-dependent steroid production. Preincubation of Leydig cells with pertussis toxin for 2 h did not affect LHRH-A or LH-stimulated pregnenolone production. It may have been possible that the effects of pertussis toxin evolved more slowly than the effects of cholera toxin.

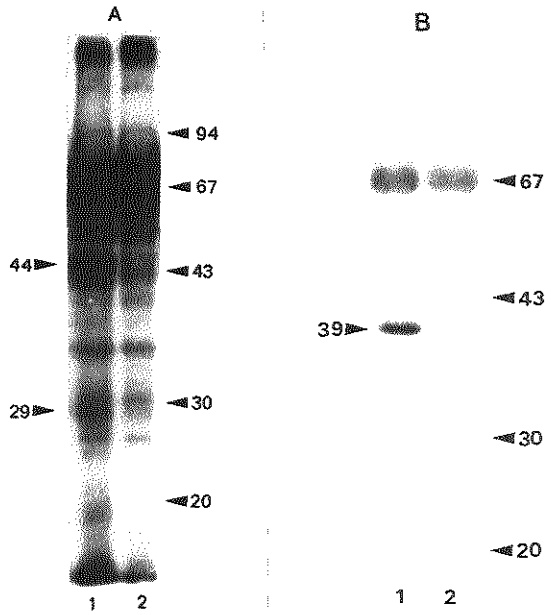

Figure 1. A. Effect of cholera toxin on ADP-ribosylation of Leydig cell membranes. A crude membrane preparation was incubated for 1 h at 30°C with (^{32}P)-NAD in the absence (lane 2) or presence (lane 1) of cholera toxin. The position of the marker proteins is indicated on the right.
B. Effect of pertussis toxin on ADP-ribosylation of Leydig cell membranes. A crude membrane preparation was incubated for 1 h at 30°C with (^{32}P)-NAD in the absence (lane 2) or presence (lane 1) of pertussis toxin. The position of marker proteins is indicated on the right.

Therefore, we investigated the effects of the toxins after a 24 h preincubation period. Cells were incubated for 24 h with cholera toxin and pertussis toxin (100 ng/ml). After this period, the medium was changed and LH or LHRH-A were added. The pregnenolone production was determined after a 3 h incubation period (figure 2). In the presence of LH the pregnenolone production was stimulated 7 fold, but LHRH-A did not have an effect. It has been shown previously that the stimulatory effect of LHRH-A on steroid production is lost after culturing the cells for 24 h (Hunter et al, 1982; Rommerts et al, 1984a). Pretreatment of the cells with cholera toxin or pertussis toxin resulted in a stimulation of both basal and LH-dependent pregnenolone production. The stimulatory efect of pertussis toxin was small when compared to the effect of cholera toxin. When cells

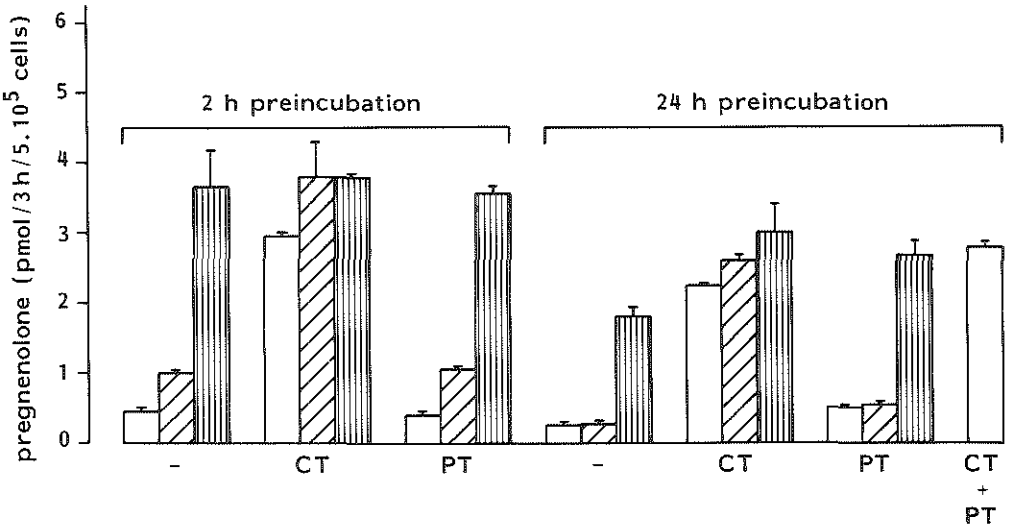

Figure 2. Effect of cholera toxin and pertussis toxin on pregnenolone production in rat Leydig cells. Cells were preincubated for 2 h (left) or 24 h (right) with cholera toxin (100 ng/ml) or pertussis toxin (100 ng/ml), and subsequently incubated for 3 h without hormones (open bars), with 40 nM LHRH-A (cross-hatched bars) or 100 ng/ml LH (striped bars). Values given are means±SD (n=3) of one cell preparation.

were treated with both toxins, the stimulatory effects of cholera toxin and pertussis toxin appeared to be additive. The steroid production in cells that were treated with LHRH-A was not different from control cells.

DISCUSSION

The presence of a stimulatory (N_s) and an inhibitory (N_i) guanine nucleotide binding protein in Leydig cells was investigated by using cholera toxin and pertussis toxin as known modifiers of these proteins. Our results indicate that cholera toxin catalyzed the ADP-ribosylation of a 44 kDa protein in a crude Leydig cell membrane preparation. The cholera toxin dependency of the labelling, and the observed molecular weight strongly suggest that this protein is the α-subunit of N_s (Kaslow et al, 1980; Lai et al, 1981; Gill, 1984). The functional importance of N_s in Leydig cells was evaluated by stimulating cholera toxin treated cells with LH or LHRH-A, and determining pregnenolone, the product of cholesterol side chain clea-

vage, which is the rate determining step of steroidogenesis in the Leydig cell. Pregnenolone production is stimulated in Leydig cells that have been preincubated with cholera toxin for 2 or 24 h. It has been shown that cholera toxin stimulates cAMP levels and testosterone production in Leydig cells (Cooke et al, 1977; Dufau et al, 1978). Moreover, it has been observed in Leydig cell membrane preparations that the stimulation of adenylate cyclase by hCG is dependent on the presence of guanine nucleotides (Dufau et al, 1980; Mukhopadhyay & Schumacher, 1985). Hence, it may be suggested that the effect of LH on steroid production is mediated by LH. Preincubation of Leydig cells for 24 h in the presence of cholera toxin resulted in a potentiation of the effect of LH on steroid production. This may have been the result of an activation of N_s-proteins which are not coupled to the LH receptor, in this way stimulating steroid production.

It has been suggested that the presence of both N_s and N_i is needed for a maximal effect of hormones on cAMP levels (Cerione et al, 1985). The molecular weight of the pertussis toxin substrate (39 kDa), and the observed decrease in the labelling of the protein when GTPγS and $MgCl_2$ were present in the incubation strongly suggests that this protein is the α-subunit of N_i (Bokoch et al, 1983; Murayama & Ui, 1983; Ribeiro-Neto et al, 1985). However, preincubation of intact Leydig cells with pertussis toxin for 2 h did not lead to stimulation of basal, LH- and LHRH-A-dependent steroid production. When pertussis toxin is present for 24 h the basal, LH- and cholera toxin-stimulated pregnenolone production was potentiated. These results are consonant with the observations of Adashi et al (1984), who showed that that pertussis toxin stimulated the hCG-dependent testosterone production in testicular cells after a 24 h preincubation period with the toxin. It may be that the effects of pertussis toxin evolve more slowly than the effects of cholera toxin. It has been observed in different cell systems that preincubation periods ranging from 60 min (Volpi et al, 1985; Holz et al, 1986) to 24 h (Adashi et al, 1984; Veldhuis & Hewlett, 1985) are necessary for pertussis toxin to exert its effects. Alternatively, the Leydig cells may have become more sensitive to pertussis toxin during culture. Similar changes in Leydig cell sensitivity during culture have been shown with adenosine (Rommerts et al, 1984) or β-adrenergic agonists (Anakwe et al, 1985). These observations indicate that N_i is involved in the regulation of steroid production in rat Leydig cells, although its mechanism of action is

not clear.

N_i may also mediate the effect of receptors for hormones that have an inhibitory effect on adenylate cyclase (Birnbaumer et al, 1985). However, we did not find effects of hormones of this type on steroid production (unpublished observations). It has been observed that the inhibitory effects of arginine-vasotocin on hCG-stimulated steroid production could be abolished by pertussis toxin, suggesting that this peptide may activate N_i-coupled receptors in testicular cells (Adashi et al, 1984). However, these authors used a testicular cell preparation containing many other cell types in addition to Leydig cells. Therefore it is not clear whether the effects of arginine-vasotocin or pertussis toxin were directly on Leydig cells, or mediated by other cell types. Furthermore, during the 11 day culture period employed by Adashi et al, (1984) the Leydig cells may have become more sensitive to arginine-vasotocin. It is not clear whether N_i is involved in the effects of LHRH on steroid production. After Leydig cells have been cultured for 24 h, LHRH inhibits the LH-dependent steroid production (Hunter et al, 1982; Rommerts et al, 1984), and it may be necessary to design experiments measuring this activity of LHRH-A to elucidate the possible involvement of N_i in the action of LHRH on Leydig cell steroidogenesis.

In conclusion, we have shown that the α-subunits of N_s and N_i are present in mature rat Leydig cells. The cholera toxin substrate N_s may be involved in LH, but not in LHRH regulation of steroid production. N_i is also involved in the regulation of steroid production in rat Leydig cells, but the mechanisms involved are not clear.

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