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THE EFFECT OF INTERLEUKIN-1α AND TESTICULAR MACROPHAGES ON ADULT RAT LEYDIG CELL STEROIDOGENESIS *IN VITRO*

by

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ABSTRACT

The project was aimed at studying the effect(s) of interleukin-1 (IL-1) and testicular macrophages on androgen release by adult rat Leydig cells *in vitro* in the context of the paracrine regulation of Leydig cell function. Purified Leydig cells were cultured with or without IL-1α, usually for 72 h, followed by 4 h in the presence of LH, dbcAMP or pregnenolone. Arachidonic acid metabolism inhibitors were also used. The medium was collected daily and assayed for androgen and, in some cases, cAMP and/or progesterone content.

IL-1α increased basal androgen release and enhanced the effect of submaximally effective concentrations of LH (less than 1 ng/ml), dbcAMP (30 μM) and pregnenolone (0.3 μM). IL-1α thus seems to stimulate steroidogenesis at a step distal to pregnenolone formation. IL-1α increased basal cAMP release but this effect occured later than the increase in androgen. Blocking the lipoxygenase pathway of arachidonic acid metabolism with NDGA resulted in a gradual but complete disappearance of this effect. Cyclooxygenase inhibitors were without effect.

IL-1α had a dose- and time-dependent inhibitory effect on the response to 100 ng LH/ml. IL-1α also inhibited the response to 30 μM dbcAMP and 3 μM pregnenolone indicating that IL-1α has an inhibitory effect on the 17α-hydroxylase/C_{17-20} lyase. LH-induced cAMP levels were also inhibited after 3 days with IL-1α suggesting the existence of more than one site of action. Lipoxygenase and cyclooxygenase inhibitors did not affect the inhibitory effect of IL-1α. Testicular macrophage conditioned medium (TMCM) had a stimulatory effect on both basal and LH-stimulated androgen release, suggesting that IL-1 is not the active factor in the TMCM.

Taken together the data suggest that IL-1α stimulates androgen release but also decreases the maximum steroidogenic capability of the cells. The data also support the hypothesis that an IL-1-like molecule and testicular macrophages are involved in the paracrine regulation of Leydig cell steroidogenesis.

LIST OF ABBREVIATIONS

aa	amino acid(s)
AA	Arachidonic Acid
ACTH	Adrenocorticotrophic Hormone
AOV	Analysis of Variance
ASA	Acetyl Salicylic Acid
AVP	Arginine Vasopressin
BSA	Bovine Serum Albumin
cAMP	3′, 5′ - Cyclic Adenosine Monophosphate
CL	Corpus Luteum
CRF	Corticotropin Releasing Factor
CSF	Colony Stimulating Factor
DAG	Diacylglycerol
dbcAMP	dibutyryl cAMP
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FSH	Follicle Stimulating Hormone

FSH/TMCM	TMCM from FSH-treated Testicular Macrophage Cultures
G-Protein	Guanosine Triphosphate Regulated Protein
GH	Growth Hormone
GnRH	Gonadotrophin Releasing Hormone
hCG	Human Chorionic Gonadotropin
HETE	Hydroxyeicosatetraenoic Acid
HPETE	Hydroperoxyeicosatetraenoic Acid
IBMX	3-Isobutyl-1-methylxanthine
IGF	Insulin-like Growth Factor
IL-1	Interleukin-1
INF	Interferon
IP_3	Inositol 1,4,5-triphosphate
kDa	kiloDaltons
LAF	Lymphocyte Activating Factor
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
LPS	Lipopolysaccharide
LT	Leukotriene
mRNA	Messenger Ribonucleic Acid
NDGA	Nordihydroguaiaretic Acid
NSE	Non-specific Esterase

OT	Oxytocin
PDGF	Platelet Derived Growth Factor
PG	Prostaglandin
pI	isoelectric focussing point
PKA	Protein Kinase A (3′,5′-Cyclic Adenosine Monophosphate-dependent Protein Kinase
PKC	Protein Kinase C (Calcium-Phospholipid Dependent Protein Kinase)
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLSD	Product of Least Square Difference
PMA	Phorbol Myristate Acetate
PRL	Prolactin
PS	Phosphatidyl Serine
PTX	Pertussis Toxin
SCC	cytochrome P450 Side Chain Cleavage enzyme complex
SCF	Sertoli Cell Factor
TGFα	Transforming Growth Factor α
TGFβ	Transforming Growth Factor β
TMCM	Testicular Macrophage Conditioned Medium
TNF	Tumour Necrosis Factor
TSH	Thyroid Stimulating Factor

U	Unit (of activity)
3β-HSD	Hydroxysteroid Dehydrogenase
17α-OH Progesterone	17α-hydroxyprogesterone
25-OH Cholesterol	25-hydroxycholesterol

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INTRODUCTION

The testis has two functions; the production of gametes through the process of spermatogenesis and the production of steroids required for spermatogenesis as well as for development and maintenance of secondary sex characteristics. These two functions take place in separate testicular compartments and are primarily controlled by pituitary hormones. It was long believed that, except for the role of testosterone in sperm production, spermatogenesis and steroidogenesis were controlled and occurred independently of each other. In recent years, it has become apparent that this is not the case. It is now well accepted that testicular function is not only regulated by pituitary hormones but also by locally produced substances which link the functions of cells in the seminiferous tubules and the interstitial compartments. While much has been learned in the past decade, the paracrine regulatory mechanisms are still poorly understood. The goal of this research project was to further our understanding of the paracrine regulation of Leydig cell function and more specifically, to study the effect of interleukin-1 (IL-1) on Leydig steroidogenesis.

Interleukin-1 was originally considered as a potential paracrine regulator of Leydig cell function because it is a major product of macrophages and the interstitial space of the testis contains a substantial population of resident macrophages. *In vitro* studies looking into the function of these testicular

macrophages have shown that they can release substances which alter Leydig cell steroidogenesis. Researchers have now demonstrated that an IL-1-like substance is being produced in the testis. However, in the normal testis, it appears to be released by the seminiferous tubules rather than by testicular macrophages. The possible release of IL-1 by testicular macrophages under pathological conditions and the possibility that IL-1 produced in the periphery during an immune challenge may reach Leydig cells has not yet been investigated. Nevertheless, the mere presence of IL-1-like activity in the testis supports the concept that IL-1 may play a role in the paracrine regulation of the function of Leydig cells.

Understanding the mechanism(s) involved in the paracrine control of testicular function requires an appreciation of the complexity of the anatomical organization of the testis. In the following pages the organization of the testis and the characteristics and function(s) of its major cell types will be briefly described. A review of the basic evidence and major findings supporting the concept of paracrine regulation of Leydig cell function as well as an overview of the substances which are being considered as local regulators will then be presented. Finally, IL-1, its biochemistry, origin and physiological role(s) will be discussed.

Anatomical and Functional Organization of the Testis

1 - The Seminiferous Tubules

In the rat, 80-90% of the total testicular volume is occupied by the seminiferous tubules which are the site of spermatogenesis (Mori & Christensen, 1980; Setchell, 1981). This testicular compartment comprises three major cell types; the myoid cells, the Sertoli cells, and germ cells at various stages of development.

a) The Myoid Cells

The myoid cells are an important part of the basement membrane of the tubules, the basal lamina. These smooth muscle-like cells possess androgen receptors and are likely responsible for the peristaltic movement of the tubules. Testosterone is required for their differentiation and stimulates their contractility. The presence of myoid cells in the basal lamina is believed to be essential for the development and function of Sertoli cells (Setchell, 1981; Waites & Gladwell, 1982). Their interactions with Sertoli cells are being actively studied.

b) The Sertoli Cells

A single layer of evenly spaced Sertoli cells lies directly against the basal lamina. Mature Sertoli cells have a columnar shape, oriented towards the centre of the tubules. Their nucleus has a pyramidal shape and their cytoplasm

contains a combination of organelles similar to that commonly seen in steroid secreting cells (Rich & de Kretser, 1983; Setchell, 1981). Sertoli cells have androgen receptors and are considered to mediate the effect of these steroids on germ cell development (Setchell, 1981). While Sertoli cells lack the cholesterol side-chain-cleavage (SCC) and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzymes, they are capable of producing testosterone from progesterone. The presence of aromatase enzyme activity has been demonstrated for the immature rat but its presence in the adult is controversial (Rich & de Kretser, 1983). Small amounts of several steroidogenic enzymes are present in the seminiferous tubules and the Sertoli cells but the level of these enzymes is much greater in interstitial cells. Because of this and the fact that androgens produced in the interstitial space can diffuse freely into the seminiferous tubules, it is felt that the only steroidogenic enzymes of physiological relevance in Sertoli cells are those involved in the metabolism of testosterone to dihydrotestosterone (DHT) and possibly estrogen, and of DHT to androstanediols (van der Molen & Rommerts, 1981).

c) The Blood-testis Barrier

Sertoli cells display several types of specialized junctions which link them to each other or to developing germ cells (Setchell, 1981). The junctional complexes between Sertoli cells which are located near the basal lamina form the blood-testis barrier and are of particular significance to testicular function.

The blood-testis barrier divides the seminiferous tubules into a basal compartment, between the basal lamina and the junctional complexes, and an adluminal compartment located above the junctions. The blood-testis barrier protects the developing germ cells from the body's immune system but most importantly it is essential for the formation and maintenance of the environment required by developing germ cells for completion of meiosis and full maturation (Setchell, 1981; Waites & Gladwell, 1982).

The seminiferous tubules are avascular (Setchell, 1981; Sharpe, 1984) and are not penetrated by any nerves (Setchell, 1981). As a result, any signals they receive, hormonal or otherwise, and any nutrient they require must be derived from the interstitial fluid. Because of the blood-testis barrier, nearly every molecule that reaches the adluminal compartment must first pass through the Sertoli cell cytoplasm. Evidence gathered over the last decade indicates that the seminiferous tubule environment is maintained both by controlling the entry of molecules into the tubules and by regulating the composition of the interstitial fluid. The latter would require the local production and release of molecules capable of altering interstitial blood vessel permeability, flow and/or Leydig cell function. Substances produced by the seminiferous tubules have been demonstrated to modify the function of cells and structures of the interstitial compartment. Those substances, as they relate to Leydig cell function, will be discussed in the next section.

d) The Germ Cells

Germs cells are found around and between Sertoli cells and migrate (or are transported) from the basal compartment to the adluminal compartment at the time when they develop surface antigens and enter meiosis. The maturation of germ cells, from stem cell to spermatozoa, occurs in an orderly and synchronized fashion. A look at cross sections of seminiferous tubules reveals that germ cells at a given state of maturation are only found in association with cells at certain other states of maturation. Because germ cells develop in synchrony, a given section of the seminiferous epithelium goes through a fixed and cyclical sequence of these cellular associations, a phenomenon called the spermatogenic cycle. While several systems of classification exist, one often used is that of Clermont & Leblond (1952) which divides the spermatogenic cycle into 14 different stages, numbered I to XIV.

The seminiferous epithelium also shows spatial organization. Adjacent sections of the epithelium are at the preceding or following stage of the spermatogenic cycle and because spermatogenesis proceeds in a synchronized fashion, this relationship is maintained. (Rich & de Kretser, 1983; Setchell, 1981; Steinberger, 1971). Of particular importance to the concept of paracrine control of testicular function is the fact that the 'nutritional' requirements of germ cells vary with their developmental stage and that it is the function of Sertoli cells to meet these needs. For instance, it has been established that germ cells at

certain stages of development are more dependent on the presence of testosterone than at other stages, probably reflecting the dependence of certain Sertoli cell functions on this steroid. Since this need for androgen is stage specific and keeping in mind the spatial organization of the germinal epithelium, the presence of a local mechanism for maintaining high steroid levels when needed would be a reasonable assumption.

2- The Interstitial Space

The interstitial space of the testis accounts for 6-16% of the testicular volume in the rat (34% in humans and around 40% in the boar) (Christensen, 1975; Mori & Christensen, 1980). This testicular compartment contains blood vessels, small nerves, lymphatic drainage and several cells types. Fibroblasts, Leydig cells and macrophages are the most noticeable cells of the interstitial tissue but occasionally T cells (Niemi, Sharpe & Brown, 1986), plasma cells (Ohata, 1979) and, in humans, mast cells (Christensen, 1975; Kerr & de Kretser, 1981) are also observed. Three main patterns of organization of the interstitial tissue have been described based on the extent of the connective tissue, the type of lymphatic drainage and the number and distribution of the Leydig cells (Christensen, 1975). One of these patterns, the one found in the rat and mouse will be emphasized in the following review.

a) The Leydig Cells

i) Structure and Spatial Organization

Nearly 70% of rat interstitial cells have been identified as Leydig cells by immunohistochemical (monoclonal CRB 15) and cytochemical (3β-HSD) staining (Niemi, Sharpe & Brown, 1986) and they occupy 17-30% of the interstitial space (Christensen, 1975; Mori & Christensen, 1980). Adult Leydig cells are polyhedral or spindle-shaped and have a large ovoid nucleus with a distinctive pattern of heterochromatin. As is typical of steroid producing cells, they have abundant smooth endoplasmic reticulum (ER), mitochondria containing both lamellar and tubular cristae, a well developed Golgi apparatus, lysosomes and some lipid inclusions. They also contain patches of rough ER, frequently interconnected with the smooth ER, peroxisomes, lipofuscin granules, microtubules, microfilaments and in some species, crystals of Reinke. (Christensen, 1975; Hodgson & Hudson, 1983; Mori & Christensen, 1980; Ohata, 1979)

Leydig cells are usually found in clusters located in close proximity to blood vessels (Hodgson & Hudson, 1983) and they appear to maintain close contact with the circulatory system. Perivascular Leydig cells are often connected to the basement membrane of the capillaries (Kuopio & Pelliniemi, 1989) and the blood vessel epithelium is very thin, discontinuous and appears

absent over a large part of the surface of the Leydig cell clusters (Hodgson & Hudson, 1983).

Most of the space around the rat and mouse interstitial cells is occupied by extensive lymphatic sinusoids (Christensen, 1975). In parallel to the blood vessels, the sinusoids lack a continuous (visceral lymphatic) endothelium and, as a result, cells from the interstitial space including Leydig cells are bathed in lymphatic fluid (Hodgson & Hudson, 1983; Fawcett et al., 1973; Miller, Bowman & Rowland, 1983). Rat Leydig cells therefore appear to be in direct communication with both the lymphatic and circulatory systems (Hodgson & Hudson, 1983). In contrast, the lymphatic drainage of the porcine and human interstitial space is provided by lymphatic vessels and the blood vessels of the human testis do not have the same thin epithelium as the blood vessels of the rat testis (Christensen, 1975). The close contact of the interstitial cells with the lymphatic and circulatory systems is therefore not present in all species and this may influence the way gonadal function is regulated, especially at the local level.

Several features of the surface of Leydig cells suggest the existence of interactions with adjacent cells. In most species, filopodia extend from the Leydig cell surface and form tight and gap junctions with filopodia from adjacent Leydig cells (Christensen, 1975; Kerr & de Kretser, 1981; Ohata, 1979).

Desmosome-like junctions between Leydig cells have also been observed (Ohata, 1979). Short processes extend from Leydig cells into invaginations of the testicular macrophage membranes. Patches of basement membrane are present on the surface of Leydig cells (Kerr & de Kretser, 1981; Kuopio & Pelliniemi, 1989; Ohata, 1979) and they may play a role in maintaining contact between adjacent Leydig cells and between Leydig cells, macrophages and capillaries (Kuopio & Pelliniemi, 1989).

ii) Steroidogenic Function

The primary function of Leydig cells is to produce steroids and in particular, androgens. They are the major site of testicular steroid production although, as was discussed above, Sertoli cells may also contribute to a small extent (Rich & de Kretser, 1983; van der Molen & Rommerts, 1981).

Testosterone is produced by the sequential action of several enzymes on cholesterol and subsequent steroid intermediates. Most of the cholesterol necessary to sustain steroidogenesis by rat Leydig cells comes from *de novo* synthesis within the Leydig cells themselves. The rate limiting enzymatic step in Leydig cell steroidogenesis appears to be the production of pregnenolone from cholesterol by the action of the mitochondrial cholesterol side chain cleavage (SCC) enzyme. However, the true rate limiting step is that regulating the availability of cholesterol to the SCC enzyme (Hall et al., 1981). This step

is regulated by trophic hormones and current evidence indicates that it requires *de novo* protein synthesis (Stocco & Soderman, 1991). Stimulation of Leydig cells by luteinizing hormone (LH) enhances SCC activity and thus pregnenolone production (Dufau, 1988; Hodgson & Hudson, 1983). This is probably the most important site of action of LH (Dufau, 1988). The next enzymatic step consists of the conversion of pregnenolone to progesterone by the 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone is then converted to 17α-hydroxyprogesterone (17α-OH progesterone) by the 17α-hydroxylase/C_{17-20} lyase. This latter step occurs very rapidly and very little progesterone accumulates in the cell (Hodgson & Hudson, 1983). The 17α-hydroxylase/C_{17-20} lyase then cleaves two carbon side-chain off 17α-OH progesterone to produce a C19 steroid, androstenedione. LH deprivation results in a loss of 17α-hydroxylase and C_{17-20} lyase activities and the 17α-hydroxylase/C_{17-20} lyase is the site of many regulatory mechanisms. Testosterone is finally formed by the reduction of androstenedione catalyzed by the 17-ketosteroid reductase/17β-hydroxysteroid dehydrogenase. This sequence of intermediates between pregnenolone and testosterone is referred to as the Δ-4 pathway and it is the prevalent steroidogenic pathway in rodents. In humans, androgen production follows primarily the Δ-5 pathway which uses the same enzymatic activities but does not involve the production of progesterone. Instead, the 3β-HSD acts to convert androstenediol to testosterone, the last step in the pathway. The major steps in the synthesis of testicular steroids and the key enzymatic

activities involved are summarized in Figure 1. The further metabolism of testosterone to estradiol-17β by the aromatase enzyme complex (Payne et al., 1987; Tsai-Morris, Aquilano & Dufau, 1985) is particularly important when studying the regulation of steroidogenesis as will be discussed below.

In addition to the involvement of LH in the control of steroidogenic enzyme activity, feedback inhibition by steroids is also present. Pregnenolone and testosterone inhibit SCC activity and testosterone also inhibits the 3β-HSD. Estrogen causes a decrease in both 17α-hydroxylase and C_{17-20} lyase activities. The desensitization of adult rat Leydig cells to LH stimulation, a phenomenon characterized by 'lesions' of the 17α-hydroxylase/C_{17-20} lyase, is believed by some (Dufau, 1988) to be the result of increased estrogen production. Aromatase inhibitors have been reported to block the desensitization of the 17α-hydroxylase/C_{17-20} lyase and fetal and neonatal rat Leydig cells, which lack the aromatase enzyme complex, do not desensitize upon sustained LH stimulation. Furthermore, the levels of 17α-hydroxylase/C_{17-20} lyase mRNA in fetal Leydig cells is decreased following estrogen exposure (Nishihara et al., 1988). Others, (reviewed in Payne, 1990) favour a depletion of endogenous cholesterol as the cause of depressed androgen production following acute LH or cAMP stimulation. Anakwe & Payne (1987) have shown that the levels of the 17α-hydroxylase/C_{17-20} lyase must be reduced to 10% or less of its pre-stimulation levels before a reduction in androgen release can be measured.

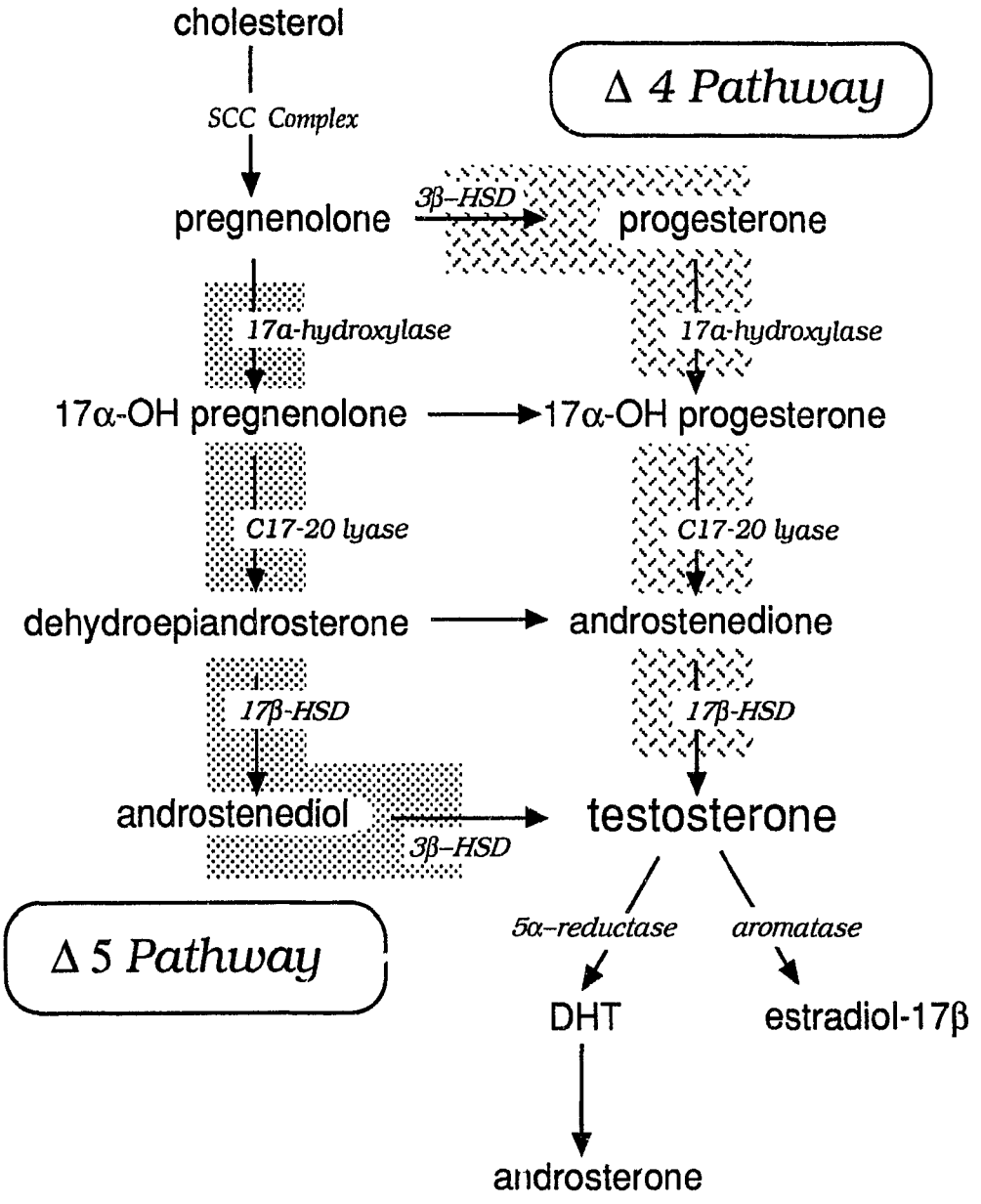

Figure 1.

Schematic representation of the Δ 4 and Δ 5 pathways leading to the formation of testosterone from cholesterol and the major metabolites of testosterone. Enzymes or enzymatic activities are depicted in italics. This diagram illustrates only the mains steps and metabolites involved.

iii) Other Leydig cell Products

Leydig cells synthesize and release a few products other than steroids. Activin (Lee et al., 1989) and corticotropin releasing factor (CRF) (Fabbri, Tinajero & Dufau. 1990; Yoon, Sklar & David, 1988) are released in the medium from Leydig cell cultures. Proopiomelanocortin derived peptides, and in particular β-endorphin, are produced by Leydig cells (Boitani et al., 1985). Substance P and methionine-enkephalin immunoreactivities have also been reported in human Leydig cells (Schulze, Davidoff & Holstein, 1987).

The function of these substances is still being established but, in the case of activin and CRF, an autocrine role has been advanced. These factors will be discussed further. There are also indications that Leydig cell factors can regulate the function of other testicular cells or structures such as blood vessel permeability.

b) Testicular Macrophages

i) Structure and Spatial Organization

The interstitial space of the testis contains a significant population of resident macrophages (El-Demiry et al, 1987; Christensen, 1975; Miller, Bowman & Rowland, 1983; Ohata, 1979) which in the rat accounts for 20% to 28% of the interstitial cell profile (Bergh, 1985; Ewing et al., 1979; Miller,

Bowman & Rowland, 1983; Niemi, Sharpe & Brown, 1986). Testicular macrophages are usually round in shape and contain a single indented nucleus, mitochondria, dispersed rough ER, a well developed Golgi apparatus, tubular vesicles and numerous lysosomes.

Murine testicular macrophages can occur singly but are often seen in association with the Leydig cell clusters (Miller, Bowman & Rowland, 1983; Niemi, Sharpe & Brown, 1986; Ohata, 1979). In humans, they are located in concentric circles around the seminiferous tubules and the blood vessels (El-Demiry et al. 1987). Numerous filopodia and lamellopodia project into the interstitial lymphatic space, the presence of which has been linked to general phagocytic activity (Carr, 1968). The surface juxtaposed to Leydig cells is mostly smooth and there are no junctional structures between macrophages and Leydig cells. There are however, unique interactions between the membrane of the two cells types: numerous short filopodia extend from the Leydig cells into invaginations (coated pits) in the macrophage cell membranes and structures typical of the 'coated membranes' often associated with receptor-mediated transport of specific ligands are present in these invaginations. A recent study has demonstrated the presence of patches of Leydig cell basement membrane in the area of the coated pits (Kuopio & Pelliniemi, 1989). Numerous coated vesicles in various stages of formation can also be seen near the macrophage surface (Kuopio & Pelliniemi, 1989; Miller, Bowman &

Rowland, 1983).

The importance and function of these coated pits in testicular function is unclear. When certain substances such as monomeric plutonium citrate or trypan blue are injected *in vivo*, they accumulate in large amounts in testicular macrophages. It has been proposed that they may enter the macrophages via the coated membranes; in blood, plutonium is bound to transferrin and transferrin can be incorporated into certain cells through a receptor-mediated mechanism. Trypan blue binds to blood protein and the dye-protein complex may enter the macrophages via a similar mechanism. It has been suggested that this process may play a role in the detrimental effect of certain substances on gonadal function (Miller, Bowman & Rowland, 1983). Another proposition is that macrophages may engulf Leydig cell cytoplasm through these coated pits (Miller, Bowman & Rowland, 1983). While this latter proposition is still speculative, these cellular associations do suggest that testicular macrophages may play a role in the regulation of Leydig cell function.

ii) Functional and Surface Characteristics

Testicular macrophages are active phagocytes. They can ingest particulate matter through a non-immune mechanism both *in vivo* and *in vitro* (Miller, Bowman & Roberts, 1984; Niemi, Sharpe & Brown, 1986;). As is characteristic of almost all mononuclear phagocytes, they have Fc receptors on

their surface (Miller, Bowman & Rowland, 1983) and Fc receptor-mediated (immune-mediated) phagocytosis has been demonstrated *in vitro* (Miller, Bowman & Roberts, 1984). Testicular macrophages also have complement receptors (Miller, Bowman & Roberts, 1984), Ia antigens (Niemi, Sharpe & Brown, 1986; Miller, Bowman & Roberts, 1984) (in humans, HLA-DR) and several other surface antigens commonly found on macrophages (El-Demiry et al., 1987; Niemi, Sharpe & Brown, 1986; in humans, Pollanen & Niemi, 1987). These characteristics suggest that testicular macrophages may have an immune-related role in the testis. As is typical of other tissue macrophages, testicular macrophages show a strong staining for the lysosomal enzymes acid phosphatase and aryl sulfatase (Miller, Bowman & Rowland, 1983) and a strong, granular staining for non-specific esterase (NSE) diffusely distributed throughout the cytoplasm (Miller, Bowman & Roberts, 1984). These enzymes are also present in Leydig cells but the pattern of staining differs (Molenaar, Rommerts & van der Molen, 1986). Unlike Leydig cells, testicular macrophages do not bind the CRB 15 monoclonal antibody and do not stain for the 3β-HSD enzyme. These markers, in combination with macrophage specific markers, have allowed the differentiation of the two cell types with much certainty (Niemi, Sharpe & Brown, 1986). A summary list of some of the cell surface and enzymatic markers used to characterize Leydig cells and testicular macrophages are given in Table 1a and 1b.

Markers	Description	Testicular Macrophage	Leydig cell	Species	Ref.
W3/25	surface Ag. on MØ, some T-cells	Yes	No	rat	6
MRC OX1	leukocyte common Ag.	Yes	No	rat	6
MRC OX6, OX17	Ia, class II MHC	Yes	No	rat	6
MRC OX3HL	Ia (monoclonal)	10-40% Yes	n/s	rat	4
MRC OX19	T-lymphocyte surface Ag.	No	No	rat	6
CRB 15	Leydig, Sertoli, Adrenal cortex, Theca (pre-ovulatory follicle), Luteal cells	No	Yes	rat	6
Leu M3, M3.9, M5, M24	Monocytes/MØ, Interdigitating, and/or dendritic cells	Yes	n/s	human	1,7
MY4	Monocytes, some granulocytes	Yes	No	human	7
HLA-DR & -ABC	B-cells, Monocyte/MØ, activated T-cells	Yes	weak HLA-ABC	human	1,7
T-6	Langerhans cells surface Ag.	No	n/s	rat	4

Table 1a.

Table of cell markers used to differentiate and/or characterize Leydig cells and testicular macrophages. Table 1b. completes this table and contains the list of references.

n/s = result not specified for this cell type in the paper

Markers	Description	Testicular Macrophage	Leydig cell	Species	Ref.
Complement Receptor	(sheep red blood cell (SRBC) rosetting assay)	Yes	n/s	rat	4
Fc Receptor	(by SRBC phagocytosis or adherence)	Yes	No	rat	2, 3, 4
Phagocytosis	Non-Immune, In vivo (Trypan blue, Plutonium citrate)	Yes	No	rat	3, 4, 6, 8
"	Immune (Fc receptor-mediated)	Yes	n/s	rat	4
3ß-HSD	Steroidogenic enzyme	No	Yes	rat	6, 8
NSE	cytoplasmic enzyme	yes, strong granular, diffuse	yes, but different reactivity	rat	4, 5, 8
Acid Phosphatase	" "	yes, like most tissue MØ	yes, but pattern different	rat	3
Aryl Phosphatase	" "	yes, like most tissue MØ	yes	rat	3

1. El-Demiry et al., 1987
2. Hutson, 1989
3. Miller, Bowman & Rowland, 1983
4. Miller, Bowman & Roberts, 1984
5. Molenaar, Rommerts & van der Molen, 1986
6. Niemi, Sharpe & Brown, 1986
7. Pollanen & Niemi, 1987
8. Yee & Hutson, 1983

Table 1b.

Table of cell markers used to differentiate and/or characterize Leydig cells and testicular macrophages. This table completes the list started in Table 1a.
n/s= cell type not specified in the reference

The absence of 3β-HSD activity in testicular macrophages and the absence of pregnenolone production by Leydig cell-free, macrophage-enriched cell preparations in response to LH or 22R-hydroxycholesterol suggest that testicular macrophages are probably not steroidogenic (Themmen et al., 1987). On the other hand, peritoneal macrophages can metabolize androstenedione to several androgens, including testosterone (Milewich et al., 1982). So, while they may not possess 'early' steroidogenic enzymes, it has not been ruled out that testicular macrophages may be involved in further processing gonadal steroids. This aspect deserves further study.

The Paracrine Regulation of Leydig Cell Steroidogenesis

1 - General Aspects

The existence of paracrine mechanisms of regulation of Leydig cell function was proposed nearly a decade ago (Bergh, 1982; de Kretser, 1982). Since then, much evidence has accumulated to indicate that not only Leydig cells but also Sertoli, myoid and germ cells are all involved in an intricate paracrine regulation of testicular function (see Saez et al., 1987; Swinnen et al., 1990).

Prior to the 1980's it was felt that since follicle stimulating hormone (FSH) modulated mostly Sertoli cell function and LH controlled Leydig cell

function and the two cell types are found in separate testicular compartments, Leydig cells and Sertoli cells must be functioning independently of each other. One of the earliest pieces of evidence that challenged this concept was the observation that focal damage to the seminiferous tubules (using silastic implants containing anti-spermatogenic agents) resulted in changes in the morphology of Leydig cells located in areas adjacent to, but not in areas distant from the point of damage where normal spermatogenesis was occurring (Aoki & Fawcett, 1978). Since Sertoli cells play a central role in the maintenance of spermatogenesis, it was felt that they probably released a factor or factors capable of altering Leydig cell function in order to optimize the availability of substances needed for sperm production (de Kretser, 1982).

Results obtained from experimental unilateral cryptorchidism are also indicative of local regulatory mechanism of Leydig cell function; while both the scrotal and abdominal testis are exposed to the same circulating gonadotropin levels, the size and the *in vivo* and *in vitro* steroidogenic properties of Leydig cells from the scrotal and abdominal gonads differ markedly (Jansz & Pomerantz, 1986; Kerr et al., 1988; Risbridger, Kerr & de Kretser, 1981). Furthermore, the changes in Leydig cell and testicular macrophage morphology associated with human chorionic gonadotropin (hCG) treatment differ between the abdominal and scrotal testis (Bergh, 1987).

Several parameters of Sertoli cell function vary depending on the stage of the spermatogenic cycle with which they are associated. For instance, changes in their response to FSH (Parvinen et al., 1980) and their production of androgen binding protein (Ritzen et al., 1981) have been detected. In addition, the local concentration of androgen in the seminiferous tubules varies with the stage of spermatogenesis, being highest at stages VII-VIII which are the stages of sperm release (Parvinen, 1982), therefore providing further evidence for some local mechanism regulating these parameters. Studies by Bergh (1982, 1983) revealed that Leydig cells adjacent to stages VII-VIII of the spermatogenic cycle, stages which have the highest requirement for androgen (Parvinen, 1982; Russel, Malone & Karpas, 1981), are substantially larger in size than Leydig cells associated with other stages. When associated with stages I-VIII, perivascular Leydig cells are smaller than peritubular Leydig cells but no difference in size is seen when they are associated with stages IX-XIV. In the abdominal testis of experimental unilateral cryptorchid rats where spermatogenesis is disrupted, there are no differences in size between any of the Leydig cells (Bergh, 1983). These studies strongly suggest that there must exist a mechanism other than changes in the release of pituitary hormones to account for such local variations in testicular function and morphology. The central role played by Sertoli cells in spermatogenesis made them a prime candidate for mediating these local changes.

The demonstration that conditioned medium from Sertoli cell cultures modulates *in vitro* Leydig cell steroidogenesis provided the first direct evidence for the involvement of these cells in the paracrine regulation of Leydig cell function (see Saez et al., 1985 and Saez et al., 1987 for reviews). Some of the active molecules contained in the spent medium have been characterized and their effect on Leydig cells will be described below. In addition, the effects of some factors released by and affecting Leydig cells will be described. An interleukin-1-like substance is also released by the seminiferous tubules but since interleukin-1 is the focus of this work, it will be discussed at greater length in a separate section. Recently, it has become evident that Leydig cells can modulate their own function through the production of autocrine agents. The effects of CRF and activin will be reviewed since they may also play a role in creating local changes in Leydig cell and/or testicular function.

2 - Intratesticular Factors Affecting Leydig Cell Function

a) Luteinizing Hormone-Releasing Hormone

The extra-pituitary actions of luteinizing hormone releasing hormone (LHRH) [also called gonadotropin releasing hormone (GnRH)] were demonstrated first for the ovary and then for the testis (reviewed in Hsueh & Jones, 1981). When a testicular source of LHRH-like activity was discovered, 'testicular LHRH' became the first putative paracrine regulator of Leydig cell function to be

identified (Sharpe et al., 1981). Testicular LHRH is believed to be secreted by Sertoli cells (see Tahka, 1986 for review). It has both stimulatory and inhibitory effects on Leydig cell steroidogenesis and the effect is dependent on the type of Leydig cell, the duration of culture and the concentration of LH used. Alone or in the presence of low concentrations of LH, LHRH has a short lived stimulatory effect. In the presence of high concentrations of LH, it is inhibitory (Hsueh, 1981; Hsueh et al., 1983; Sharpe, 1984; Sharpe & Cooper, 1987; Tahka, 1986). Specific LHRH receptors are present in postnatal Leydig cells (Hsueh et al., 1983) and their numbers increase during maturation. They are not detectable on freshly isolated fetal cells but they are present after a few days in culture. LH decreases and LHRH treatment increases LHRH receptor number (Dufau, 1988).

The effect of LHRH appears to be species specific. While its influence on rat Leydig cells function is well documented, LHRH has no effect on normal and transformed mouse Leydig cells and human Leydig cells (Ascoli, Euffa & Segaloff, 1987; Cooke & Sullivan, 1984; Verhoeven & Cailleau, 1986). High affinity LHRH receptors have only been identified on rat Leydig cells (see Rommerts & Themmen, 1986 for review)

b) Factors Present in Seminiferous Tubules and Sertoli Cell Culture Conditioned Medium

While much data indicate that the seminiferous tubules can modulate Leydig cell morphology and steroidogenesis, both stimulatory and inhibitory activities have been obtained and it is still unclear which of these effects predominates *in vivo*. Sertoli cells are generally considered the source of these 'activities' but, because Sertoli cell cultures are usually contaminated to a certain extent with other seminiferous tubule cells, researchers are cautious about concluding on the exact source of the factor they are studying. Because several factors could be present in the conditioned medium from Sertoli cells or seminiferous tubule cultures, it is often unclear whether the effect of such conditioned medium on Leydig cells represents the action of one or many 'factor(s)'.

Several groups have reported that the conditioned medium from seminiferous tubule cultures contains a factor which inhibits basal (Vihko & Huhtaniemi, 1989) and LH-induced Leydig cell steroidogenesis (Syed et al., 1989; Vihko & Huhtaniemi, 1989) as well as LH- and forskolin-induced cAMP accumulation (Vihko & Huhtaniemi, 1989). This inhibitory factor does not alter LH binding and seems to act by inhibiting adenylate cyclase directly. Its secretion begins around the time of puberty, can be advanced by FSH treatment (Syed et al., 1989) and is influenced by the stage of spermatogenesis

(Syed et al., 1989; Vihko & Huhtaniemi, 1989). It is not released by tubules from cryptorchid animals but prenatal testicular irradiation, which destroys germ cells and thus abolishes spermatogenesis, does not affect its secretion. This indicates that germ cells are not the source of this inhibitory factor but that certain types of spermatogenic disruption, like those seen in cryptorchidism, can influence its release (Syed et al., 1989).

Factors with stimulatory activity have also been obtained from seminiferous tubules or Sertoli cell-enriched cultures. Sertoli cell-enriched cultures from prepubertal testes release a factor which stimulates the early steps of the steroidogenic pathway in prepubertal Leydig cells while inhibiting the conversion of C21 to C19 steroids (Verhoeven & Cailleau, 1985). Its release is increased by FSH suggesting that it originates from Sertoli cells rather than from contaminating cells. Its effect is additive to that of LHRH and, contrary to the inhibitory tubular factor described above, it has no effect on cAMP accumulation. Interestingly, this factor is not specific to Sertoli cells cultures and is also secreted by numerous permanent cells lines (Verhoeven & Cailleau, 1985). This non-specificity does not rule out a paracrine role for this factor since its release may be regulated by changes in the local environment. Recently, the spent medium from adult seminiferous tubule or Sertoli cell cultures has been shown to stimulate basal and LH-stimulated estradiol and testosterone secretion by adult rat Leydig cells despite causing a decrease in

cAMP. The factor(s) is not LHRH and is not controlled by FSH or testosterone (Carreau, Papadopoulos & Drosdowsky, 1988; Papadopoulos et al., 1987b) unlike the stimulatory factor described by Verhoeven & Cailleau, (1985).

Immature seminiferous tubules release a factor that inhibits aromatase activity in adult rat Leydig cells and it may be that this factor is involved in the low aromatase activity seen in immature Leydig cells (Papadopoulos et al., 1987a).

c) Transforming Growth Factor α and Epidermal Growth Factor

Epidermal growth factor (EGF) and transforming growth factor α (TGFα) share sequence homology and bind to the same receptor (Marquart et al., 1984). The effect of EGF on Leydig cell function has been studied more extensively than that of TGFα but recent data suggest that they may both be important in the regulation of testicular steroidogenesis.

EGF is secreted by the submaxillary salivary gland in larger amounts in males than in females and its release is influenced by testosterone. Consequently, the effect of EGF on testosterone release has received much attention. Leydig cells have EGF receptors and EGF has both inhibitory and stimulatory effects on steroidogenesis, depending on the system in which it is tested. In normal and transformed Leydig cells (Ascoli et al., 1987; Dufau, 1988) EGF

exposure causes a down-regulation of LH-receptor and inhibits 17α-hydroxylase/C_{17-20} lyase activity (see Dufau, 1988 and Bellvé & Zheng, 1989 for reviews). Recently, a stimulatory effect of EGF, occurring independently of cAMP formation, has been reported for adult and immature Leydig cells and tumour Leydig cells (Ascoli et al., 1987; Verhoeven & Cailleau, 1986). The amount of EGF released *in vivo* is high enough to potentially induce the effects observed *in vitro*. EGF may therefore be a peripheral extra-pituitary factor involved in the control of gonadal function.

Unlike EGF, data indicate that there is an intra-testicular source of TGFα. A TGFα-like protein is present in the testis (Skinner et al., 1989) and it shares several features of the Sertoli cell derived growth factor recently described by Holmes et al. (1986). In fact, it is currently felt that they may be one and the same. Immunoreactive TGFα accumulates in the culture media of Sertoli cell cultures and TGFα mRNA is present in Sertoli cells and myoid/peritubular cells. Peritubular cells respond to and have receptors for TGFα (Skinner et al., 1989). Immunoreactive TGFα has recently been identified in Leydig cells but it is not clear at this time whether this represents the uptake of TGFα by Leydig cells from the interstitial fluid or production of TGFα by Leydig cells. Based on information from other cells types, the latter possibility is favoured (Teerds, Rommerts & Dorrington, 1990). TGFα is present in all fetal and adult Leydig cells but only in some prepubertal cells. It is during the prepubertal

period that adult Leydig cells begin to emerge, probably though differentiation of mesodermal cells. It has been proposed that the differentiating cells have the appearance of Leydig before they have acquired all the functional characteristics of mature Leydig cells, including immunoreactive TGFα. TGFα may thus prove to be a useful marker of Leydig cells maturation (Teerds, Rommerts & Dorrington, 1990).

TGFα is produced as a membrane-anchored protein but it can be cleaved off and released as a free extra-cellular protein. Both forms may have biological importance. The membrane-bound form is active and thought to be involved in cell-cell interactions. If it occurs in that form in Leydig cells, it could conceivably be involved in the membrane interactions observed between filopodia from adjacent Leydig cells. If TGFα is present in its free form, it could function as a paracrine or autocrine factor (Teerds, Rommerts & Dorrington, 1990). Leydig cells have EGF receptors and respond to EGF (described above) and, since TGFα uses the same receptor as EGF, it is likely that Leydig cells can respond to TGFα in the same manner.

d) Inhibin, Activin and Transforming Growth Factor β

The polypeptide chains of TGFβ (a homodimer) shares sequence homology with the β chain of inhibin (αβ heterodimer) and activin ($\beta_A\beta_B$ dimer) and with Mullerian inhibiting factor (homodimer). These factors are all members

of a same gene family (Sporn et al., 1986).

TGFβ has an inhibitory effect on Leydig cell steroidogenesis in culture. It inhibits gonadotropin-induced androgen release (Fauser, Galway & Hsueh, 1988; Lin, Blaisdell & Haskell, 1987; Morera et al., 1988) and it suppresses hCG-induced cAMP accumulation either through its effect on LH/hCG receptor number (Avallet et al., 1987; Morera et al., 1988) or through a direct inhibition of adenylate cyclase (Avallet et al., 1987). TGFβ also has an inhibitory effect at a site distal to cAMP formation (Lin, Blaisdell & Haskell, 1987). TGFβ exerts a similar inhibitory effect on adrenal steroidogenesis (Rainey, Viard & Saez, 1989) but has a stimulatory action on ovarian cells (Fauser, Galway & Hsueh, 1988). The increase in the conversion of pregnenolone to testosterone in the presence of TGFβ indicates that it exerts its inhibitory effect at a step prior to pregnenolone metabolism (Avallet et al., 1987). In immature porcine Leydig cells, low doses of TGFβ have a stimulatory effect on testosterone production (Morera et al., 1988). Messenger RNA for TGFβ is present in porcine Sertoli cells and Sertoli cell conditioned medium contains TGFβ-like activity. This TGFβ-like activity is probably responsible for the inhibitory activity of Sertoli cell conditioned medium previously described (Avallet et al., 1988; Perrard-Sapori et al., 1987)

Inhibin is released by Sertoli cells under the control of FSH (Bicksak et al., 1987). and is better known as an inhibitor of FSH release. It may, however, participate in the paracrine regulation of Leydig cell function since it enhances gonadotropin-stimulated testosterone production by Leydig cells *in vitro* (Fauser, Galway & Hsueh, 1988).

Activin stimulates FSH release and has effects similar to those of TGFβ on Leydig cells (Fauser, Galway & Hsueh, 1988). Maudiuit and coworkers (1991) recently reported that both activin A ($\beta_A\beta_A$) and TGFβ have a stimulatory effect on the 3β-HSD enzyme which is probably mediated by a common mechanism. In addition, activin A has a partial inhibitory effect on the SCC enzyme. The cellular origin of activin in the testis has not been fully established but a recent study indicates that Leydig cells may be its source (Lee et al., 1989). If that is the case, activin would act more as an autocrine factor. However, it is not yet clear whether Sertoli cells can also release activin. Sertoli cells synthesize both the α and β chains needed to produce inhibin (αβ) and whether they also assemble the ββ dimer remains to be demonstrated (Lee et al., 1989). Recent data are suggesting that TGFβ, inhibin and activin may also be involved in the regulation of gonadal cell growth (Gonzalez-Manchon and Vale, 1989).

e) Insulin-Like Growth Factor

Insulin-like growth factor-1 (IGF-1), also called somatomedin C, is present in the testis in amounts comparable to those of major IGF-1-producing organs such as the liver, lungs and kidney (see Bellvé & Zheng, 1989 for review). It is released in the medium of Sertoli cell cultures (Smith et al., 1987) and the amount released is increased by treatment with growth hormone, FSH, EGF or fibroblast growth factor (FGF) (see Bellvé & Zheng, 1989 for review). Specific IGF-1 receptors have been identified on Leydig cells (see Bellvé & Zheng, 1989 for review) and their number is increased by *in vivo* hCG, FSH or GH treatment (Lin, Blaisdell & Haskell, 1988). Insulin and IGF-1 have similar effects on rat Leydig cells and it has been suggested that insulin may exert its stimulatory effect on steroidogenesis by acting through the IGF-1 receptor (Majercik, Czerwiec & Puett, 1989).

Chronically or as a pretreatment, IGF-1 stimulates LH/hCG- or cAMP analog-induced testosterone production, possibly through the maintenance of hCG receptor number (Bernier et al., 1986). However, since IGF-1 also stimulates the conversion of pregnenenolone to testosterone, another site of action may exist (De Mellow, Handelsman & Baxter, 1987; Lin, Blaisdell & Haskell, 1988; Moger & Murphy, 1987). A stimulatory effect on basal testosterone production has also been noted (see Lin, Blaisdell & Haskell, 1988; Bellvé & Zheng, 1989). According to a recent report, IGF-1 stimulates aromatase activity

in adult rat Leydig cells but not in immature porcine Leydig cells (Rigaudière, Grizard & Boucher, 1989). This effect is somewhat inconsistent with the overall stimulatory effect of IGF-1 on testosterone production. As previously discussed, estrogen has been implicated in the desensitization of Leydig cells to LH stimulation and in the negative modulation of the 17α-hydroxylase/C_{17-20} lyase. One would thus expect that a factor stimulating aromatase activity would also trigger the estrogen-induced inhibition of 17α-hydroxylase/C_{17-20} lyase.

A lack of effect of IGF-1 on mouse interstitial cells has been reported and may indicate species specific differences in responsiveness to IGF-1 (Bebakar et al., 1990). IGF-1 has no effect on the MA-10 cell line, which is derived from mouse Leydig cell tumor, and whether this is a species related effect or due to the low level of late-steroidogenic enzyme activities characteristics of those cells is unclear (Majercik, Czerwiec & Puett, 1989).

While testicular IGF-1 is released by Sertoli cell cultures, the possibility that testicular macrophages may also be able to produce it may be worth investigating. Lung macrophages can release an IGF-1-like molecule upon activation. Since both lung and testicular macrophages are resident tissue macrophages, testicular macrophages may also release IGF-1-like material when activated (Rom et al., 1988).

f) Corticotropin Releasing Factor

A corticotropin releasing factor (CRF) molecule, identical to hypothalamic CRF (see Dufau, 1988), is present in the testis of many species (Audhya et al., 1989). It is released by and affects the function of Leydig cells (Fabbri, Tinajero & Dufau, 1990). Therefore CRF, like activin, appears to be an autocrine regulator of Leydig cell function. CRF inhibits hCG-induced cAMP and steroid production by adult and fetal rat Leydig cells (Fabbri, Tinajero & Dufau, 1990; Ulisse, Fabbri & Dufau, 1989) and basal steroid production in adult rat cells (Ulisse, Fabbri & Dufau, 1989) through a specific receptor (Ulisse, Fabbri & Dufau, 1989). The release of CRF appears to be regulated by at least two second messenger systems since it is increased by hCG, cAMP analogs, forskolin and by protein kinase C activation.

g) Arginine-Vasopressin

Specific V_1 arginine-vasopressin (AVP) receptor sites are present on Leydig cells and an AVP-like molecule is present in large amounts in the testis of several species (Kasson, Adashi & Hsueh, 1986; Tahri-Joutei & Pointis, 1989). The production of testicular AVP-like peptide appears to be regulated independently from pituitary AVP. This has been elegantly demonstrated by showing that Brattleboro rats, which lack pituitary AVP due to a genetic defect, have normal testicular AVP (Kasson & Hsueh, 1986).

Arginine-vasopressin (AVP) stimulates basal androgen release by adult rat Leydig cells and by prepubertal, pubertal and adult mouse Leydig cells. This effect is short lived and is absent after 24h of treatment (Sharpe & Cooper, 1987; Tahri-Joutei & Pointis, 1989). An inhibitory effect on hCG-stimulated androgen release is also present in adult rat and in pubertal and adult mouse Leydig cells but it is seen only after 24 to 72h of treatment. AVP and arginine-vasotocin (AVT) (both have similar antigonadal potency) inhibit hCG- and dibutyryl cyclic AMP (dbcAMP) stimulated testosterone production by Leydig cells from hypophysectomized rats without altering hCG binding. In this system, AVP inhibits the 17α-hydroxylase/C_{17-20} lyase enzyme but appears to stimulate the cholesterol SCC enzyme since basal testosterone production is not changed by AVP treatment but basal pregnenolone and progesterone production are increased (reviewed in Kasson, Adashi & Hsueh, 1986).

Hypophysectomized adult rats, whose spermatogenesis is maintained with FSH, respond to *in vivo* AVP treatment with a decrease in hCG-binding capacity, hCG- and dbcAMP-stimulated androgen release and *in vitro* forskolin-induced cAMP accumulation. A profound suppression of spermatogenesis accompany these changes (Adashi, Resnik & Zirkin, 1987). Paradoxically, intratesticular AVP injections in normal mice and rats have failed to alter Leydig cell steroidogenesis (Tahri-Joutei & Pointis, 1989). Thus, the role testicular AVP in the regulation of testicular function remains unclear.

h) Oxytocin

Oxytocin (OT) is present in the testis of several species. Some evidence indicates that Leydig cells may be the source of testicular OT which would act to stimulate seminiferous tubule contractility (Dufau, 1988) but there are also reports of an effect of OT on Leydig cell function. OT has been found to increase basal androgen release by mouse Leydig cells (pre- to postpubertal) but not by cells from adult rats. No effect on hCG-stimulated androgen release has been reported for normal rat and mouse Leydig cells but an inhibition of hCG-induced testosterone release has been observed with mixed testicular cells from hypophysectomized rats (Adashi, Tucker & Hsueh 1984). OT binds to the AVP receptor and this may be how it exerts its action (Tahri-Joutei & Pointis, 1989). When present, the effect of OT on Leydig cells is small and its significance has not been established.

Interleukin-1

Interleukin-1 (IL-1) was originally described as a protein released by activated macrophages acting as a co-mitogen of T lymphocytes (Gery, Gershon & Waksman, 1972) and, accordingly, was named 'lymphocyte activating factor' or LAF. The term 'interleukin' was proposed and adopted in 1979 when it was realized that the large number of factors derived from cells of the immune system and named after particular biological effects on lymphoid or inflam-

matory cells, represented only a few molecules with diverse effects. The macrophage factors previously termed LAF, B cell activating factor and B cell differentiation factor (see Mizel, 1989 for review) were then grouped under the name 'interleukin-1'. There are now at least eight different 'interleukins'. The biological activity of purified or recombinant IL-1 is still often measured in terms of its effect on T-lymphocytes and thus expressed as 'LAF units'.

1 - Biochemistry of IL-1

There are several forms of IL-1 which can be classified by their molecular weight (MW), isoelectric focusing point (pI) and even by their range of biological activities. The MW of polypeptides with IL-1 activity ranges from 2 to 75 kilodaltons (kDa) with the smaller MW probably representing proteolytic fragments, and the large MW (75 kDa) an aggregate form. The pI of 'IL-1' molecules ranges from 4.0 to 8.0. The two major forms of IL-1, known as IL-1α and IL-1β, are synthesized as large (~31 kDa) precursor molecules and are post-translationaly processed to the ~17 kDa mature form. Their pI are approximately 5.0 and 7.0 for IL-1α and IL-1β, respectively.

The first IL-1 gene to be cloned was that coding for murine, pI 5.0 IL-1 pro-peptide (Lomedico et al., 1984). The resulting protein had 270 amino acids (aa) and no signal peptide. The gene for human pro-IL-1 with a pI of 7.0 was cloned shortly after (Auron et al., 1984) and coded for a 269 aa protein, also

lacking a signal peptide. In 1985, March and coworkers cloned a second human pro-IL-1 gene, the gene coding for the pI 5.0 form of IL-1. This group identified the two forms of IL-1 as coming from separate genes and proposed to name the pI 5.0 form IL-1α and the pI 7.0 form IL-1β. The precursor proteins from various species have around 270 aa while the mature proteins range from 152 to 159 aa. It has been established that bioactivity resides in the C-terminal end of the molecule (March et al., 1985). A point mutation of the IL-1β gene, resulting in the substitution of amino acid 127 of the mature peptide from arginine to glycine, results in a 100-fold reduction in bioactivity with only small changes in receptor binding affinity (Gehrke et al., 1990). The two IL-1 genes have now been located on chromosome 2 (Webb et al., 1986) and, despite low nucleotide homologies (~45%) (March et al., 1985), they have very similar structure and organization. Each gene contains 7 exons of similar length and positioning of the intron/exon boundaries (Clark et al., 1986). Clark et al. (1986) have proposed that these similarities are "consistent with both common ancestry as well as functional constraint on evolutionary diversification". It is estimated that the pro-IL-1 gene dates back 450-700 million years, prior to the elaboration of a true immune system (Clark et al., 1986). This may explain why IL-1 and related molecules can be produced by so many different cells types and have such diversified biological activities (see below). There is evidence that IL-1α and IL-1β are members of a family of functionally related proteins (Martin & Resch, 1988; Oppenheim et al., 1986) and the presence of a possible

alternate splicing site on the pro-IL-1β gene (Clark et al., 1986) suggests that some of these proteins may also be structurally related. The amino acid sequence homology between human IL-1α and IL-1β is only 26% and, in other species varies from 22% to 26% homology (Mizel, 1989). The sequence for a given form of IL-1 is highly homologous between species, however. For example, rat pro-IL-1α is 65% homologous to human pro-IL-1α, 64% homologous to rabbit pro-IL-1α and 83% to murine pro-IL-1α (Nishida et al., 1989). As a result, IL-1α is active across species (Nishida et al., 1989).

The lack of a leader or signal sequence in the IL-1 peptide is peculiar and has left scientists puzzled about the mechanism by which IL-1 is moved outside of the cells. IL-1 is not secreted through the classical 'endoplasmic reticulum - Golgi - secretory vesicles' route. The cleavage of the IL-1 precursors does not necessarily occur intracellularly and does not appear to be required for secretion. The precursor and the mature protein are released from activated monocytes in culture with the same kinetics suggesting that the same secretory mechanism is used for both molecules (Hazuda et al., 1988) but the nature of this mechanism is still unclear.

The precursor and mature form of IL-1α are equally bioactive (Mosley et al., 1987). Most reports indicate that pro-IL-1β must be processed before exerting its effect(s) (Black et al., 1988; Hazuda et al., 1988; March et al., 1986;

Mosley et al., 1987) but some conflicting reports exist regarding that point (Jobling et al., 1988). Macrophages can release the enzyme(s) needed to cleave the pro-IL-1 molecule along with the pro-IL-1. Enzymes produced locally by other cells can also cleave the pro-IL-1 molecules and, particularly in the case of IL-1β since this form probably requires processing to gain biological activity, this may be of significance in inflammatory diseases (Hazuda et al., 1990). In humans and mice, IL-1β is expressed to a greater extent than IL-1α (5:1, Clark et al., 1986) and the presence of an additional promotor-like sequence for the IL-1β gene may be the cause of this difference. In addition, the IL-1α gene has a poor TATA box consensus sequence and no obvious CAT box (Clark et al., 1986). The efficiency of the promotor for the pro-IL-1β gene has been estimated to be 10-50 times greater than that of the pro-IL-1α gene promotor (March et al., 1985). It may be that the synthesis of biologically active IL-1α and IL-1β are regulated differently; gene transcription for IL-1α and post-translation processing for IL-1β.

2 - The IL-1 Receptors and Signal Transduction

Understanding the mode of action of IL-1 has proven to be difficult and much still remains to be clarified. Part of the difficulty comes from the fact that IL-1 can trigger several second messenger systems. Depending on the cell type, IL-1 has been shown to increase cAMP release (Muñoz et al., 1990; Shirakawa et al., 1988; Turunen, Mattila & Renkonen, 1990) and/or hydrolysis of various

membrane phospholipids (Kester et al., 1988; Mochan et al., 1989; Rosoff, Savage & Dinarello, 1988; Wijelath et al., 1988; Zawalich & Zawalich, 1989). IL-1 has also been shown to be internalized with its receptor and to subsequently localized within the cell nucleus where it is believed to exert some effect (Curtis et al., 1990). In some cell types more than one system may be activated and the final biological response depends on the interactions between systems (Mochan et al., 1989; Muñoz et al., 1990).

The multiplicity of intracellular signalling cascades triggered by IL-1 goes hand-in-hand with the existence of several classes of receptor. At least two separate receptors exist and there may be more. The best characterized receptor was first purified from the EL-4 cell line, a T-cell derived line, and it has since been cloned (Sims et al., 1988). This receptor has a molecular weight of 80-85 kDa (before deglycosylation) and binds both IL-1α and IL-1β (Bird and Saklatvala, 1986; Dower et al., 1986; Kilian et al., 1986). Reported affinity (K_d) for the IL-1 receptor on EL-4 cells ranges from about 3×10^{-10}M to 4×10^{-9}M (Curtis et al., 1990; Dower et al., 1986; Horuk & McCubrey, 1989; Kilian et al., 1986; Sims et al., 1988). Lowenthal & MacDonald, 1986 have identified two separate receptor types on EL-4 cells, They detected a high number (200-20,000) of 'lower' affinity sites ($K_d = 5 \times 10^{-10}$M) and a low number (12-400) of high affinity sites ($K_d = 5 \times 10^{-12}$M). Others have failed to detect the high affinity sites on EL-4 cells (Bomsztyk et al., 1991; Sims et al., 1988) but there is a report

of such high affinity receptors on lung fibroblasts (Chin et al., 1987). The presence of two separate receptors on T cells is supported by the recent findings of Muñoz et al. (1991) which indicate that IL-1 activates two separate second messenger systems in the D10A cell line and that blocking the 80 kDa IL-1 receptor blocks the induction of only one messenger system. The number of IL-1 receptor per cell varies from a few hundreds to 30,000 sites, depending on the cell type (Lowenthal and MacDonald, 1986; Dower et al., 1985; Kilian et al., 1986; Matsushima et al., 1986). The cloning of the 80 kDa IL-1 receptor present on EL-4 has revealed that it is a member of the superfamily of immunoglobulin genes which is characterized by its 3-dimensional structure and its organization into constant and variable regions. Other members of this superfamily include the CD4, CD8, MHC (major histocompatibilty complex) molecules and PDGF and CSF1 (colony stimulating factor 1) receptors. The intracellular portion of the receptor has no significant homologies with known sequences and its sequence did not reveal its signal transduction mechanism. There is, however, a sequence that resembles an acceptor site for a protein kinase C and phosphorylation at this site may play a role in receptor function (Sims et al., 1988).

B-cells, macrophages and bone marrow granulocytes carry IL-1 receptors that is (or, are) a different gene product than the IL-1 receptor found on T-cells, fibroblasts and epithelial cells (Chizzonite et al., 1991). At this time, the

consensus is that B-cells carry a 60-70 kDa IL-1 receptor while T-cells, fibroblast and epithelial cells carry the 80-90 kDa receptor. The K_d of the B-cell type IL-1 receptor is around 1-5 x 10^{-10}M. Contrary to the T-cell IL-1 receptor, the receptor on B-cells does not appear to downregulate (Horuk & McCubrey, 1989). An additional IL-1 binding site of greater affinity (K_d = 6 x 10^{-11}M) may also be present in low number (~100 sites/cells) on B-cells (Bomsztyk et al., 1991). The presence of receptor subunits which could explain some of the differences in receptor size and affinity, as well as the induction of different second messenger systems, has not been ruled out yet.

It is worth mentioning that IL-1 receptor antagonists have been identified and a recent report looking into their distribution in the brain points out that they may play an important role in controlling the final biological impact of IL-1 (Licinio et al., 1991).

3 - Biological Effects of IL-1

Despite their limited amino acid homology (26% for human IL-1s; March et al., 1986), IL-1α and IL-1β usually bind to the same receptor (Dinarello et al., 1989) and induce similar biological response in a wide variety of cells (Dinarello, 1988a, 1988b). During the inflammatory or immune response, IL-1 is released primarily by macrophages and can affect the function of a broad range of non-lymphoid target cells (Martin & Resch, 1988), including

fibroblasts, synovium cells, hepatocytes, muscle cells, osteoclasts and chondrocytes as well as cells from the central nervous system through which it induces fever (Dascombe et al. 1989). IL-1 can also alter the function of, and be produced by many non-lymphoid cell types (Dascombe et al., 1989; Martin & Resch, 1988) and thus may play a much broader role in the regulation of physiological processes than initially foreseen (Blalock, 1989; Kordon & Bihoreau, 1989). IL-1 mRNA has been detected in normal blood leukocytes (Dinarello, 1985), as well as normal spleen, kidney, liver (Tovey et al., 1988), noradrenergic chromaffin cells (Schultzberg et al., 1989) and nerve fibres associated with numerous organs (Schultzberg et al., 1987). Plasma IL-1 is elevated after ovulation in women (Cannon & Dinarello, 1985) and amniotic fluid is also high in IL-1-like activity (Tamatani et al., 1988). It has even been suggested that IL-1 should be considered a hormone since it has systemic effects and it can be present in cells and biological fluids under 'normal' conditions (Oppenheim et al., 1986). Amongst the non-immune function of IL-1, and of particular interest in the frame of the work presented here, is a direct effect on several endocrine glands. IL-1 has been shown to inhibit thyroid stimulating factor (TSH) induced thyroglobulin release and to stimulate thyrocyte proliferation (Kawabe et al., 1989; Yamashita et al., 1989), to stimulate insulin release by the β-cells of the pancreas (Molvig et al., 1986; Spinas et al. 1986; Zawalich & Zawalich, 1989), directly stimulate steroid release by the adrenal cortex (Roh et al., 1987; Winter et al., 1990), although probably not

through a direct effect on the cortical cells but rather by stimulating intra-adrenal regulatory mechanism (Andreis et al., 1991), to inhibit FSH-induced granulosa cell differentiation, basal and LH-induced progesterone release and granulosa cell luteinization (Fukuoka et al., 1988; Gottschall et al., 1987; Mori et al. 1989) and, as will be discussed in a later section of this thesis, it modulates testicular steroidogenesis (Calkins et al.,1988; Fauser, Galway & Hsueh, 1989; Moore & Moger, 1991; Verhoeven et al., 1988). In addition, IL-1 has been shown to stimulate the release of CRF by the hypothalamus (Berkenbosch et al., 1987; Sapolsky et al., 1987) and to directly stimulate the release of adrenocoticotrophic hormone (ACTH), LH, TSH, GH but inhibit prolactin (PRL) release by the anterior pituitary (Bernton et al., 1987). *In vivo* intraventricular injection of IL-1 has a somewhat different effect on pituitary hormone release in that it inhibits LH and TSH release while stimulating GH and PRL (Rettori et al., 1987; Rivier & Vale, 1989). IL-1 may therefore modulate adrenal, thyroid and gonadal function both directly and through an effect on the hypothalamus and anterior pituitary. Rivier & Vale (1989) have recently demonstrated that IL-1 is able to affect *in vivo* testicular steroidogenesis by a direct gonadal effect as well as via a pituitary effect.

Goals of the Research Project

The goals of this research project were to further our understanding of the paracrine regulation of testicular steroidogenesis. The presence of macrophages in the interstitial space of the testis and the reported effect of testicular macrophage culture conditioned medium on Leydig cells steroidogenesis were the original premises that prompted the study of the effects of IL-1, a major macrophage product, on Leydig cells. It was subsequently reported that an IL-1-like factor was released by cells from the seminiferous tubules rather than by testicular macrophages. While this finding took the study away from the study of the paracrine role of testicular macrophages it does supports the concept that IL-1 is a paracrine regulator of testicular steroidogenesis. Furthermore, the possibilities that testicular macrophages can release IL-1 under certain 'pathological' conditions and that IL-1 from the periphery can affect Leydig cell function both remain to be explored. There is an ever growing body of evidence indicating that the immune and endocrine systems feedback or influence each other significantly. This increases the impact of the findings regarding IL-1 and Leydig cell function.

This thesis will describe the observed effects of recombinant human IL-1α on basal and stimulated androgen release by adult rat Leydig cells in primary cultures. Time course, reversibility and dose dependence of the effects

of IL-1 will be described along with studies on the site of action of IL-1 in the steroidogenic pathway. The mode of action of IL-1 was also studied and those results will be presented. Finally, preliminary studies on the effect of testicular macrophages on Leydig cell steroidogenesis will be briefly described and discussed in the context of the paracrine regulation of Leydig cell function.

MATERIALS AND METHODS

Materials

Medium 199, collagenase (type I), bovine serum albumin (BSA, type V), insulin, fetal bovine serum (FBS, hybridoma tested), dimethylsulphoxide (DMSO), vitamin E (dl-α-tocopherol acetate), sodium bicarbonate ($NaHCO_3$), HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid), l-glutamine, pyruvate, 25-hydroxycholesterol (25-OH-cholesterol), pregnenolone, progesterone, dibutyryl cAMP (dbcAMP), 3-isobutyl-1-methylxanthine (IBMX), calf thymus DNA, bisbenzamide (Hoechst 33258), acetyl salicylic acid (ASA), indomethacin, nordihydroguaiaretic acid (NDGA), *E. coli* lipopolysaccharide (LPS) and pertussis toxin (PTX) were all purchased from Sigma Chemical (St-Louis, MO, USA). All the other chemicals used for the preparation of the various assay buffers were also obtained from Sigma. Percoll and Dextran T-70 were obtained from Pharmacia Chemicals (Dorval, Québec, Canada) and Gibco (Burlington, Ontario, Canada) was the supplier for penicillin-streptomycin (PenStrep) and the Nunc-Star tubes used in the progesterone assay. Activated charcoal was obtained from BDH Chemicals (Toronto, Ontario, Canada) and testosterone from Steraloids Inc. (Whilton, NH, USA). Dr P. Lomedico from Hoffman-LaRoche (Nutley, NJ, USA) generously provided the human recombinant interleukin-1α (IL-1α) (1×10^7 U/mg) used in most experiments. Recombinant human IL-1β a preparation of human IL-1α was purchased from Collaborative Research (Waltham, MA). These IL-1 preparations were used to

the verify if different types or preparations of IL-1 all had the same effect on Leydig cells. Luteinizing hormone (LH) (NIH-LH-B9; 0.7 x NIH-LH-S1) and follicle stimulating hormone (FSH) (NIAMDD ovine FSH-14) were a gift from Dr L. Reichert Jr. and the NIAMDD Pituitary Hormone Distribution program University of Maryland, School of Medicine, Baltimore, MD, USA).

Tritiated testosterone ([^{3}H] T; [1,2,6,7-^{3}H testosterone) and the iodinated progesterone conjugate ([^{125}I] P4; progesterone-11α-hemisuccinate-(2-[^{125}I] iodo-histamine) were purchased from Amersham (Oakville, Ontario, Canada). The [^{3}H]T was stored as a stock solution in benzene/absolute ethanol (9:1, v/v) at 4°C (4.4 x 10^7 dpm/ml while the progesterone trace was stored in progesterone assay buffer at 4C (25 x 10^4 dpm/ml) and used within 4 weeks of initial analysis by Amersham. The cyclic AMP assay kits were procured from Biomedical Technologies Inc. (Stoughton, MA, USA). The iodinated cAMP contained in the kit was dissolved in the acetate buffer supplied and stored at 4°C. The expiry dates on the kits were respected.

Isolation and Culture of Leydig cells

Adult Sprague-Dawley rats (275 - 300g) were obtained from Canadian Hybrid Farm (Centreville, Nova Scotia, Canada) and were housed in the animal care facilities. The animal were cared for and handled in compliance with the guidelines of the Canadian Council on Animal Care (CCAC). The

euthanesia procedure used also conformed to the CCAC guidelines.

Rats were killed by CO_2 asphyxiation. The rate of delivery of the CO_2 was kept low to avoid stressing the animal. Immediatly after the death of the animals the testes were removed and immersed in ice-cold sterile saline (0.9% (w/v) NaCl). The dissecting equipment was kept immersed in 70% ethanol to minimize contamination. In general, no more than 6 rats were sacrificed at once as it was found that using more rats increased the time period where the cells were left to sit in the various solutions, resulting in lower steroid release.

The culture medium (medium 199) was prepared according to the manufacturer's specifications and supplemented with 0.1 mM pyruvate, 27 mM $NaHCO_3$, 10 mM HEPES and 100 mg/ml l-glutamine. The medium was sterilized by positive pressure filtration through 0.22 µM filters and stored at 4°C in sterile bottles for less than 2 weeks. When BSA was added to the medium, it was added to a few hundred millilitres of medium, thouroughly mixed and filter sterilized into the rest of the medium.

From this point onward, the procedure was performed in a 'clean air' hood with sterilized equipment. The testes were perfused with approximately 1.5 ml ice-cold sterile saline through the testicular artery, decapsulated and the artery dissected out. The prepared testes were then placed in a 50 ml conical,

plastic centrifuge tube containing a solution of ice-cold collagenase (135 U collagenase/ml culture medium plus 5 mg BSA/ml, 7 ml per 2 testes) and kept on ice until all testis were prepared. Four testes were placed in each tube. The tubes were placed in a shaking water bath oscillating at approximately 80 cycle per minute, at 32°C for 22 min. This time period was determined in preliminary experiments as giving the best tissue dispersion without compromising the steroidogenic function of the cells. Rat Leydig cells are very sensitive to damage. Rommerts et al. (1985) have demonstrated that the amount, type and even the batch of collagenase can have a profound influence on the steroidogenic response of rat Leydig cells *in vitro*. In addition, rat Leydig cells are very sensitive to mechanical damage and can be negatively affected by the frequency of the shaking water bath and the duration of the incubation. During the early phase of this research project, these difficulties with the isolation of Leydig cells were experienced and the procedure optimized to circumvent them. It is our experience that even excessive agitation of the cells during re-dispersion of the cell pellet can be detrimental to the subsequent performance of the cells.

Once the digestion period was completed, the tubes were filled with ice-cold medium 199 containing 1 mg BSA/ml (medium + BSA), gently mixed, placed on ice and the seminiferous tubules and other large particles allowed to settle for 5 min. The supernatant was filtered through 2 layers of nylon mesh

(44 and 72 μm pore size) into a new, sterile 50 ml conical tube. The tube was then filled to the 50 ml mark with medium + BSA and centrifuged for 5 min at 260 x g in a refrigerated (5^0C) centrifuge (PR-6000, Damon/IEC Division, Needham Hts. Ma, USA). The supernatant was discarded, 3 ml (per 4 testes) of medium + BSA was added to the tube and the pellet gently dispersed by rotating the tube. The two-step Percoll gradients were prepared using the one-step method provided by Pharmacia; for each gradient, 35 ml of 80% Percoll was prepared by mixing 3.5 ml 10 X HEPES (100 mM HEPES, 1.45 M NaCl, 50 mM KCl, 50 mM glucose, 10 mM $CaCl_2$, pH 7.4) with 31.5 ml medium + BSA. Twelve millilitres of 80% Percoll was then diluted 1:1 with medium + BSA to obtain the 40% layer. Twenty millitres of 40% Percoll was poured into the conical tube and 20 ml of 80% Percoll was layered underneath it with a 30 ml syringe fitted with a 3 inch long, 16 gauge needle (cathether needles, Critikon Canada Inc. Markham, Ont. Canada). The cell suspension (3 ml per gradient) was layered over a two-step Percoll gradient (Kuhn-Velten et al., 1982) using a sterile glass Pasteur pipette. The tip of the pipettes had been flamed to smooth the edges and minimise damage to the cells (Laws et al., 1985). The gradients were centrifuged for 15 min. at 420 x g at 5°C. In this system the Leydig cell layer migrates to the interface of the two Percoll layers (40-80%; densities 1.06 and 1.11 g/cubic cm, respectively). The Leydig cells with the best responsiveness to gonadotropin stimulation have been shown to have a density of 1.059 to 1.09 g/cubic cm (Laws et al., 1985; Rommerts et al.,

1985) and thus would be found at the bottom of the 40% Percoll layer. To recover the cells, the top layer of the gradient was removed with a pipette and the Leydig cell layer aspirated with a 30 ml syringe fitted with a 3 inch long 16 gauge needle. The needle was removed from the pipette before dispensing the cells into a 50 ml tube containing some cold medium + BSA to reduce the shearing force on the cells. The tubes were then filled to the 50 ml mark with more cold medium + BSA, gently mixed and centrifuged at 260 x g for 15 min. The supernatant was discarded, the pellet re-suspended in 10 ml of medium + BSA and centrifuged again for 5 min. The washing procedure was repeated once more except that the cells from all tubes were pooled in order to obtain a homogeneous suspension of cells. After this last wash, the cell pellet was resuspended in 2 ml (per 2 rats) of medium + BSA and placed on ice. The number of cells was counted using a haemocytometer. Sperm cells were excluded from the count.

This purification procedure was adapted from Anakwe, Murphy & Moger (1985), and Rommerts et al. (1985) and yielded cultures containing 80-85% Leydig cells as assessed by 3β-hydroxysteroid dehydrogenase activity (Molenaar, Rommerts & van der Molen, 1986). The staining procedure is described below. Macrophage contamination of the freshly purified Leydig cell preparation was between 7 and 9% as assessed by binding of the rat macrophage-specific antibodies ED2, ED-3 and TRPM-1 (Dijkstra et al., 1985;

Takeya et al., 1989). This procedure was performed by the laboratory of Dr. G. Rowden from the Department of Pathology at Dalhousie University.

The cell pellet was then re-suspended in Medium 199 supplemented with insulin (5 µg/ml), streptomycin (100 µg/ml), penicillin (100 U/ml), vitamin E (200 µmol/L), DMSO (100 mmol/L) and BSA (1 mg/ml). DMSO was used both as an antioxidant (Murphy & Moger, 1982) and as a solvent for the vitamin E. Vitamin E has been shown to improve the survival and gonadotropin responsiveness of porcine Leydig cells and other cell types in culture. It is probable that this effect is due to the antioxidative property of vitamin E (Mather et al., 1983). BSA was omitted in the final 4 h culture period when cells were prepared for cAMP determination to reduce protein interference in the cAMP assay. The dilution of the cell suspension was adjusted to yield 10^5 cells/200 µl. The cells were plated in Falcon Multiwell (24-well) plates (200 µl of cell suspension/well) and sufficient supplemented medium was added so to have a final volume of 1 ml per well, including the volume contributed by the treatment(s) used in the experiment. The plates were incubated at 34°C in a 95% air, 5% CO_2 humidified atmosphere for the duration of the experiment.

The spent medium was collected every 24 h, under sterile conditions, in small labelled sample pots and frozen (-20°C) until radioimmunoassayed for

androgen (Moger, 1980), cAMP and/or progesterone concentration. Fresh supplemented medium and the appropriate treatment(s) were added and the plates returned to the incubator. At the end of the culture period, the medium was replaced once more, along with the required treatment and the cells further cultured for 4 h in the presence or absence of LH, dbcAMP, 25-OH cholesterol or pregnenolone.

Isolation of Testicular Macrophages

Testicular interstitial cells were prepared as described in the isolation procedure for Leydig cells except that no BSA was added to the medium used for washing or culturing the cells. After they were counted, the cells were spun out once more and re-suspended in the volume of medium 199 containing 20% sterile calf serum (non-heat inactivated) and Pen-Strep (100 µg streptomycin /ml; 100 U penicillin/ ml) required to obtain a final concentration of cells of approximately 15 x 10^6 / ml. One millilitre of the cell suspension was placed on rectangular (24 x 30 mm; 720 mm^2) LUX cover slips which had been placed in multiwell plates (8-well plates). The plates were placed under an inverted plastic tub with a gas intake, flushed with 5% CO_2 and incubated at room temperature for 1 h. At the end of the incubation, non-adherent cells were removed by vigorously flushing the surfaces of the cover slips with warm medium 199 using a sterile wash bottle. The number of cells attached to the cover slips was estimated by counting 5 randomly selected areas on the cover

slips using an inverted field microscope fitted with an ocular with a 5 x 5 grid (0.0676 mm^2). The total number of cells counted in the 5 areas of the cover slip was divided by surface area of the 5 areas (5 x the surface of a grid; 5 x 0.0676 mm^2 = 0.338 mm^2) and then multiplied by the surface of the cover slips (720 mm^2). The cover slips were placed in new plates with 3 ml medium 199 supplemented as for Leydig cells except for the BSA and incubated in a humidified atmosphere of 5% CO_2 for 24 h. At the end of the culture period, the medium was removed, pooled in sterile 50 ml conical tubes and stored frozen at -70°C until needed. The non-purified interstitial cell preparation contained an average of 8.5% macrophages (range = 2 - 15%, as assessed by *in vivo* trypan blue phagocytosis, described below) and after purification by adherence, the cell layer contained 90.9% macrophages (range = 84.5 - 97%, by *in vivo* trypan blue phagocytosis, non-specific esterase staining, *in vitro* latex bead phagocytosis; described below). This isolation procedure resulted in the adherence of an average of 80,500 cells per cover slip. Contamination with Leydig cell was evaluated by measuring the level of androgen in the testicular macrophage conditioned medium (TMCM). This amount was always very small but always subtracted from the amount of androgen in the medium from Leydig cells cultures done in TMCM. For the co-cultures, the macrophage cell layer was cultured for 24 h in medium 199 containing the usual supplements (except for BSA) and 1% FBS. Leydig cells were purified the following day. The macrophage layer was washed with warm medium and placed in clean culture

dish. The purified Leydig cells (200,000 cells in 0.5 ml) were layered over the macrophage layer and allowed to settle for 15 min. in the incubator. An additional 1.5 ml of supplemented medium was then added to the culture dishes and the cell returned to the incubator. The rest of the culture proceeded as previously described. No BSA was used in the culture medium for these experiments. The control group consisted of Leydig cells being plated on clean cover slip and cultured in the same conditions as the co-cultures. As described for the Leydig cell cultures, the medium was changed every 24 h until the end of the experiment at which point the response to LH was assessed. In a few co-cultures, the culture medium contained either 1% FBS or 100 ng/ml LPS. The effect of these agents alone on Leydig cells was measured.

Androgen Assay

The samples were assayed for total androgen by radioimmunoassay using the antibody B-8, a generous gift from Dr. D. Armstrong (University of Western Ontario, London, Ontario, Canada). This antibody was raised against testosterone but cross-reacts extensively with other androgens (testosterone, 100%; 5α-dihydrotestosterone, 98%; 5α-androstane-3α,17β diol, 58%; 5α-androstane-3β,17β diol, 24%; androstenedione, 1.6%; androsterone, 1.2%; estradiol, 0.16%; progesterone, <0.001%; cortisol, <0.007%). Therefore the results express total androgen content of the culture media rather than testosterone content. The assay was done on unextracted samples according to the procedure

described by Anakwe & Moger (1986). As previously mentioned, the [^{3}H] T was stored in a benzene/absolute ethanol solvent. A sufficient aliquot of this solution was air dried and re-dissolved in enough steroid assay buffer (0.1M Phosphate Buffer: 154 mM NaCl, 61.4 mM Na_2HPO_4, 38.6 mM $NaH_2PO_4.H_2O$, 100 mg/L thimerosal and 1 g/L gelatin, pH 7.4) to obtain 12,000 cpm per 200 µl. The B-8 anti-androgen antibody was obtained in a lyophilized form and a 0.67% solution (w/v in 0.9% NaCl) was prepared (antibody stock solution) and frozen in small aliquots at -70°C. For the assay, the antibody stock solution was diluted 1:250 (v/v) in steroid buffer. A testosterone standard stock solution (100 ng testosterone/ml) was previously prepared and used for all assays in order to reduce interassay variability. For the assay, a standard solution was prepared by diluting the stock solution 1:100 (v/v) in steroid buffer (usually, 50 µl:5 ml. Final concentration = 1 pg testosterone/µl). All samples and standards were assayed in duplicate in 12 x 75 mm glass tubes. The assay comprised tubes for measuring total [^{3}H] T counts added to the tubes (TC = [^{3}H] T only), non-specific binding of the [^{3}H] T to the coated charcoal (NSB = [^{3}H] T + Charcoal) and binding in the absence of any unlabelled steroid (Total binding or B_0 = [^{3}H] T + Antibody + Charcoal). The standard curve included tubes to measure binding in the presence of 10, 25, 50, 100, 200, 300, 400 and 500 pg testosterone per tube ([^{3}H] T + Standard + Antibody + Charcoal). Ten or twenty five microlitres of samples were assayed, depending on their expected androgen content. Basal androgen release during

24 h of culture and LH-stimulated androgen release during 4 h period were usually measure from 10 µl aliquots while 25 µl was used for 4 h basal androgen release.

The assay was set up as follows and details on the specific amounts of each solution used in each tube can be found in Table 2. On day 1, the required amounts of [^{3}H] T, antibody and standard solutions were prepared, the tubes were placed in labelled racks and the samples were thawed and mixed. The required amount of steroid buffer was dispensed to the tubes, followed by the standards, the sample, the antibody and, finally the [^{3}H] T solution. At this point, the total volume in each tube was 800 µl. The tubes were vortexed, covered and stored at 4°C until the next day. On day 2, a solution of charcoal-Dextran was prepared (250 mg charcoal + 25 mg dextran T-70 in 40 ml steroid buffer), placed in an ice bath and allowed to mix on a magnetic stirrer for 15 minutes. The assay tubes were then taken out of the refrigerator, placed in an ice bath and 200 µl of the ice-cold, stirring, charcoal-dextran solution dispenced in each tubes except the TC tubes. The tubes were vortexed and incubated, on ice, for 10 min. The incubation was terminated by centrifuging the tube at 1000 x g for 15 min at 4°C. The antibody-bound androgen (bound) was separated from the charcoal-bound androgen (free) by decanting the supernatant into 7 ml scintillation vials. Five millilitres of scintillation cocktail (Formula-963 Aquous Counting Cocktail, NEN Research

TUBE TYPE	STD	SAMPLE	BUFFER	Ab B-8	[H] T	CHARCOAL
TC	...	...	800 µl	...	100 µl	...
NSB	...	...	600 µl	...	▽	200 µl
--STANDARDS--						
0 (Bo)	...	...	500 µl	100 µl	100 µl	200 µl
10 pg/tube	10 µl	...	490 µl			
25 pg/tube	25 µl	...	475 µl			
50 pg/tube	50 µl	...	450 µl			
100 pg/tube	100 µl	...	400 µl			
200 pg/tube	200 µl	...	300 µl			
300 pg/tube	300 µl	...	200 µl			
400 pg/tube	400 µl	...	100 µl			
500 pg/tube	500 µl	...	...	▽	▽	▽
--SAMPLES--						
'Low' Samples	...	25 µl	475 µl	100 µl	100 µl	200 µl
'High' Samples	...	10 µl	490 µl	▽	▽	▽

Table 2.

Table detailing the set up procedure for the androgen radioimmunoassay. The type of solutions used are specified in the first row and the 'tube type' is described in the first column. The volume of each solution to be dispensed in each type of tube is specified in the body of the table. 'Low' and 'high' sample refer to samples expected to contain low and high concentration of androgen, respectively.

Ab = Antibody NSB = Non-specific binding
STD = Standard TC = Total counts

Products, Boston, MA, USA) was added to the vial which were then capped and mixed. The vials were placed in a scintillation counter (1215 Rackbeta II Liquid Scintillation Counter, Wallac Oy, Finland) and the amount of bound [^{3}H] T was counted for 2 min.

The androgen content of the samples were extrapolated from the standard curve using the computer program RIAPC. The program transforms the raw counts for the standard curve according to the following equation:

$$\textbf{logit (Y)} = \log [100/(100 - \textbf{\% bound Y})]$$

% bound Y = 100 x [(cpm Y - cpm NSB)/(cpm B_0 - cpm NSB)]

Y = the standard being calculated.

Specific binding for the assay, **(cpm B_0/cpm TC)x100**, was around 40%. The assay sensitivity, corresponding to **90% B_0**, was approximately 8 pg/tube.

Progesterone Assay

The progesterone content of the samples was measured by solid phase radioimmunoassay. In brief the assay is based on coating special tubes with the progesterone antibody and counting the amount of iodinated progesterone bound to the tube via the antibody.

The assay requires the preparation of the following buffers:

50 mM Phosphate Buffer Saline (PBS):

8 mM $NaH_2PO_4 \cdot H_2O$

42 mM Na_2HPO_4

154 mM NaCl

Steroid Assay Buffer (SAB):

1 L PBS Buffer

1.0 g Gelatin

0.1 g Sodium Azide

First Coating Buffer, pH 9.4 (1st CB):

15 mM Na_2CO_3

35 mM $NaHCO_3$

0.1 g/L Sodium Azide

Store at 4C for up to two weeks.

Second Coating Buffer (2nd CB):

0.1% BSA in FBS (w/v)

Washing Buffer (WB):

0.1% Tween 80 in PBS (w/v)

The monoclonal progesterone antibody was obtained from Dr. B.D. Murphy (University of Saskatchewan, Saskatoon, Saskatchewan) and recognized the progesterone conjugate progesterone-11α-hemisuccinate-(2-

[^{125}I] iodohistamine ([^{125}I] P4) as well as progesterone. This iodinated product was obtained from Amersham, and upon arrival was diluted to obtain a stock solution of approximately 25 x 10^5 dpm/ml (8.9 ml SAB added to 10 µCi) and stored at 4°C. The trace was used, as recommended by the supplier, within 4 weeks of the initial analysis. The antibody was obtained in a lyophilized form and diluted 1:100 in distilled water. This stock solution was aliquoted and store at -70°C until needed. At the time of the assay the antibody stock was diluted in 1st CB to obtain a final dilution of 1:13,000. The radioactive trace ([^{125}I] P4), was diluted to obtain approximately 20,000 cpm/100 µl. The amount of radioactivity in the diluted trace was measured before being used in the assay. The progesterone standards were obtained by preparing a stock solution of progesterone of 800 ng/ml ethanol and further diluting it 1:100 in SAB at the time of the assay to obtain the first standard of 400 pg/50 µl. Serial dilutions of 1:2 were performed to obtain the other standards of 200, 100, 50, 25, 12.5 and 6.25 pg/50 µl.

Each samples and standards was assayed in triplicate. The details of the volume of each solution contained by each tube can be found in Table 3. On the day of the assay a fresh solution of antibody was prepared from the stock solution (final dilution 1:13,000 in 1st CB). Nunc Star tubes were labelled and the antibody dispensed into all tubes except TC and NSB tubes. The tubes were incubated either at room temperature for 90 min or overnight at 4°C. Following

TUBE TYPE	Ab	STD	2nd Coating Bf	SAMPLE	[I] PROG.	BUFFER
TC	...	...	...	...	100 µl	...
NSB	...	...	500 µl	...	⇩	150 µl
–STANDARDS–						
0 (Bo)	250 µl	...	500 µl	...	100 µl	150 µl
6.25 pg/tube		50 µl		...		100 µl
12.5 pg/tube				...		
25 pg/tube				...		
50 pg/tube				...		
100 pg/tube				...		
200 pg/tube				...		
400 pg/tube	⇩	⇩	⇩	...	⇩	⇩
–SAMPLES–						
'Low Samples	250 µl	...	500 µl	100 µl	100 µl	50 µl
'High' Samples	⇩	...	⇩	50 µl	⇩	100 µl

Table 3.

Table detailing the set up procedure for the progesterone radioimmunoassay. The type of solutions used are specified in the first row and the 'tube type' is described in the first column. The volume of each solution to be dispensed in each type pro tybe is given in the body of the table. 'Low' and 'High' sample refer to samples expected to contain low and high concentration of progesterone, respectively.

Ab = Antibody
NSB = Non-specific binding
PROG. = Progesterone
TC = Total counts

the incubation, the tubes were decanted by inversion and the remaining liquid was removed by vigourously taping the inverted tubes on layers of paper towels. The 2nd CB was added to all tubes, except TC and the tubes incubated for 45 min. at room temperature. They were then decanted as above. The standards and the samples were then added to their respective tubes followed by the addition of [^{125}I] P4 and SAB to all tubes. More SAB was dispensed in the B_0 and NSB tubes to compensated for volume of standards/sample added to the other tubes. The tubes were incubated for 4 h at room temperature after which they were decanted, except for the TC tubes, as described above. Five-hundred microlitres of wash buffer was dispensed to all tubes, except TC tubes, and were again decanted. This washing procedure, aimed at removing free [^{125}I] P4, was repeated twice more.

The amount of [^{125}I]P4 bound to the antibody was then measured by counting the tubes in a Gamma Counter (LBK-Wallac Clinigamma 1272, Finland) for 2 mins. The standard curve was calculated and the P4 content of the samples extrapolated with the RIAPC program as described for the testosterone assay. The sensitivity of this assay (90% B_0) was 40-60 pg/ml with a B_0 ranging from 16-23%.

Assay of Cyclic Adenosine-3′, 5′-monophosphate (cAMP)

The levels of cAMP that accumulate in the culture medium during a 4 h period was measured using a commercial radioimmunoassay kit. The levels of cAMP in the cells were also measured initially to establish that medium cAMP gave a fair estimate of the total amount of cAMP present in the culture. The method used to extract cAMP from the cell homogenate is detailed at the end of this section. In order to maintain continuity between experiments, the experiments where cAMP levels were to be measured were not conducted in the presence of a phosphodiesterase inhibitor. As suggested in the assay procedure, such an inhibitor (IBMX, final concentration = 500 μM) was, however, added to the medium at the time of its collection into sample pots to inhibit further degradation of cAMP. IBMX was prepared as a concentrate and added in 10 μl to each sample pot. The capped pots were vigourously shaken to optimize the mixing of IBMX. The samples were then frozen at -70°C and stored at -20°C until used. At the time of the assay, the sample were thawed, dispensed to the prepared tubes and immediatly acetylated (according to the procedure, and using the chemicals supplied with the kit) to denature any phosphodiesterases. The assay kit also contains a phosphodiesterase inhibitor. The last 4 h incubation period of these experiments was conducted with medium where BSA was omitted. This was done to reduce the interference of proteins with the assay.

The cAMP RIA assay kit utilizes a double antibody system where the second antibody is dispensed along with the radioactive tracer ([^{125}I] cAMP; [^{125}I] succinyl cAMP-tyrosine methyl ester) to simplify the procedure. The assay is very specific for cAMP. According to the manufacturer the assay is 31,000 times more specific for cAMP than for cGMP. Other related molecules (AMP, ATP, ADP) cross react even less.

The assay was set up by first preparing the various solutions needed. The tracer (1.5 μCi at the time of analysis) was provided as a concentrate and diluted with 10.5 ml of the tracer diluent provided. The latter was a sodium acetate buffer, pH 6.2, containing the second antibody (rabbit IgG), phosphodiesterase inhibitors (unspecified) and sodium azide. The assay buffer was also supplied in a concentrated form and was diluted with distilled water to obtain a 0.05 M sodium acetate buffer, pH 6.2, containing 0.01% sodium azide. The cAMP standards were prepared by adding 5 ml to the vial (final concentration = 1 nmol/ml). The cAMP-specific antibody (sheep anti-rabbit IgG) was supplied ready for use. A non-specific binding (NSB) reagent was also included, ready for use. The appropriate amounts of the reagent for acetylation (acetic anhydride and triethylamine, included) were mixed immediatly prior to their use.

The assay was performed in 12 x 75 mm glass tubes. The standard curve

was done in duplicates but only one aliquot was assayed for each sample. The standard curve was obtained by diluting the initial standard with acetate buffer in order to obtain the desired concentrations. For acetylated samples, the standards were 0.05, 0.1, 0.5, 2, 5, 10 pmol/ml. Assay buffer (100 µl) was added to the NSB and B_0 (no standard) tubes followed by 100 µl of each standards in the appropriate tubes. The samples were then dispensed in their tubes (10 µl or 100 µl) and immediatly acetylated. The tracer was added to all tubes, the cAMP antibody to all tubes but the TC and NSB tubes and the NSB reagent to the NSB tubes. When less than 100 µl of samples were assayed, enough buffer was added to compensate. The tubes were gently rotated, covered and incubated at 4°C for 18-20 h. One millilitre of assay buffer was then added to each tube (except the TC tubes which were set aside) and the tubes were centrifugated for 20 min at 1000 x g at 4°C. The supernatant was removed by inverting and blotting the tubes and the tubes counted in a gamma counter for 1 min.

For the measurement of intracellular cAMP levels the cell layers was extracted using a procedure adapted from Steiner (1974) and Gilman and Murad (1974). The culture medium was removed and 0.5 ml of cold 7% TCA (trichloroacetic acid) was added to each well. The plates were frozen at -70°C to break the cell membranes, thawed out and the TCA pipetted out into glass tubes. The wells were washed once with 0.5 ml TCA. The tubes were covered,

spun out (1000 x g at 4°C) and the TCA supernatant pipette out into glass extraction tubes. TCA was removed by extraction with water saturated ether (3 times with 5 ml ether). The aquous fraction, containing cAMP was then air dried and redissolved in 1 ml of the acetate buffer used for the cAMP assay. The extracted samples were assayed right away. The results showed that only a little cAMP remains in the cells compared to what is found in the culture medium, and that the changes in intracellular cAMP levels are similar to those seen in medium cAMP. Therefore, this extraction procedure was not used routinely. Rather, all cAMP levels reported here were measured in culture medium.

The assay was calculated as described for the androgen assay and the appropriate dilution factors were applied to obtain the amount of cAMP in the samples. The percent binding of the assay was around 40% and the sensitivity (90% B_0) ranged from 0.04 to 0.07 pmol/ml.

DNA Assay

IL-1 is know to stimulated mitogenesis in certain cell types and to be cytotoxic to others in some conditions. In order to verify if IL-1 was having an effect on Leydig cell number, the amount of DNA in the cultures, before and after IL-1 treatment was assessed. The procedure used was that reported by Labarca & Paigen (1980) with only slight modifications. This assay is based on

the ability of the fluorescent dye bisbenzamide (Hoechst 33258) to bind to DNA and to have an enhanced fluorescence once bound. Most of the assay was done under low light to reduce dye bleaching.

Several buffers and stock solutions were required for this assay and their composition is listed below. All buffers were prepared with distilled water.

Wash buffer (pH 7.4):

0.15 M NaCl

5 mM Na_2HPO_4

Phosphate buffer (pH 7.4):

50 mM Na_2HPO_4

2 mM EDTA

0.02% sodium azide

NaCl/Phosphate buffer (pH 7.4):

4 M NaCl

50 mM Na_2HPO_4

2 mM EDTA

0.02% sodium azide

Dye stock solution:

200 µg Bisbenzamide / ml H_20

Stored wrapped in foil at 4°C for up to 5 months

DNA stock solution:

500 µg calf thymus DNA / ml H_2O

Stored in a glass tube at 4C for up to 5 months

The 'true' DNA content of this stock solution was measured (on a diluted sample of the stock) by spectrophotometry (260 nm) and according to the following formula:

mg DNA/ml = (optical density x dilution)/18.5

where 18.5 = E_{260} for 1 mg calf thymus DNA / ml and optical density = $E_{260} \times C_{stock} \times 1/\text{dilution}$

For the assay, the cell layer (usually plates of cells kept frozen at -70°C) was washed with 500 µl of wash buffer and 500 µl of phosphate buffer added to the well. The cells were then sonicated for 10 s at 20 watts and a 200 µl aliquot of this extract was placed in disposable cuvettes.

The DNA standard curve was obtained by diluting the DNA stock solution 1:100 and pipetting 10, 20, 50, 100 and 200 µl in the cuvettes to obtain a curve ranging from 50 to 1000 ng DNA per cuvette. A blank cuvette, containing only the buffers and dye was also included. The volume in all cuvettes was brought to 1.5 ml with NaCl/phosphate buffer. A fresh solution of bisbenzamide was prepared by diluting the stock 1:100. Five hundred

microlitres of the dye was pipetted in each cuvette. Repeated pipetting through Pasteur pipettes was used to mix the content of the cuvettes. The samples are stable for at least 16 h if kept cold and out of the light and on occasion the samples were stored in those conditions overnight and read the next day. The samples were read in a spectrofluorometer set as follows:

excitation = 356 nm

emission = 458 nm

slit width = 10

The amount of DNA in the samples was extrapolated from the standard curve which was calculated by linear regression analysis using the computer program GraphPad Instat (Intuitive Software for Science, San Diego, CA, USA). The appropriate dilution factors were applied. The volume of sample assayed was adjusted so their DNA content fell in the mid-range of the standard curve.

Staining Procedure for 3β-hydroxysteroid Dehydrogenase

The presence of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was used to differentiate Leydig cells from macrophages since the latter have been shown to lack this enzyme (Niemi, Sharpe & Brown, 1986; Yee & Hutson, 1983). The staining procedure described here was derived from that used by Molenaar, Rommerts & van der Molen (1986).

Leydig cells were purified as described above and cultured for at least 24 h. The medium was removed and the cells stained as follows; a solution of 0.003% 5α-androstane-3β-ol-17-one, 0.017% Nitro Blue Tetrazolium, 0.017% nicotinamide and 0.017% nicotinamide adenine dinucleotide (NAD) was prepared in 0.1M phosphate buffer pH 7.1 (the buffer was prepared according to the description given in the androgen assay procedure except that no gelatin was included and the pH was adjusted to 7.1 instead of 7.4) containing 3% Dextran 400. All percentages represent a weight per volume ratio. A few drops of the staining solution was added to the cell layer which was then frozen at 70°C for about 30 min to induce damage to the cell membranes since intact cells cannot take in the dye. The cells were thawed out, a few more drops of dye solution was added and the plates incubated for 1 h at 34°C. The percentage of stained cells (blue in color) was estimated by counting cells with an inverted microscope fitted with a grided ocular (see macrophage isolation procedure). Eighty to eighty five percent of the cells stained positive for 3βHSD activity.

Staining Procedure for Non-Specific Esterase Activity (NSE)

Both Leydig cells and testicular macrophages stain for NSE. It is, however, possible to differentiate them to a certain extent by taking advantage of the difference in esterase activity levels in the two cell types. As described by Molenaar, Rommerts & van der Molen (1986), 30 seconds staining time

stains almost exclusively Leydig cells. This was supported by the 95% overlap between NSE and 3β-HSD staining in Leydig cell preparations. The staining procedure described here was derived from that published by Cooke et al. (1981) and was used for both Leydig cell and macrophage preparation with the exception that Leydig cells were stained for 30 seconds and macrophages for 20 mins.

The following staining solution was prepared fresh from stock solutions (concentration of stock solutions is indicated in parenthesis) of each component of the procedure. Eight hundred microlitres each of $NaNO_2$ (4% w/v in H_2O) and paranosaniline (2.5% w/v in 2N HCl, filtered after mixing) were mixed with 500 µl α-naphthylacetate (1% w/v in 50% acetone) and 20 ml Na_2HPO_4 (0.15 M). The staining solution was added to the wells (1.1 ml per well) along with 1 ml of medium and the plates were incubated for 20 min. or 30 s, depending on the cell type, at 34°C in 5% CO_2. The reaction was stopped by adding 1 ml of 70% ethanol to the well. The medium-stain was removed from the well, fresh medium added and the percentage of stained cells estimated as described above. For Leydig cell cultures, an average of 69% of the cells stained positively (61-77%) while approximately 90% of the cells in macrophage cultures were NSE positive. This latter number would, obviously include both macrophages and contaminating Leydig cells. However this result is similar to that obtained with both *in vivo* and *in vitro* phagocytosis (91%). Furthermore,

Leydig cells do not readily adhere to surfaces and would have been removed by the vigourous washing procedure. As previously stated, the contamination of macrophage cultures by Leydig cells was monitored by measuring the amounts of androgen released by macrophage cultures.

In vivo Phagocytosis Measurements

The ability of macrophages to phagocytise particulate matter is well recognized. This property was taken advantage of and used to easily monitor the efficacy of the macrophage isolation procedure. Rats were injected intraperitoneally with a 1 ml of 3% trypan blue solution (w/v in sterile saline) per 100 g body weight. A 3 cc syringe fitted with a 27 gauge, 1/2 inch needle was used for the injection. The rats were sacrificed 24 h later and the macrophage isolation procedure performed as described. It should be noted that if too long a period of time is allowed to elapse between the injection and the cell isolation, the macrophages become very full of trypan blue particle and attach poorly. This phenomenon has been described and excessive trypan blue phagocytosis was shown to depress macrophage function (Leblanc & Russell, 1981; Mosier, 1981). The percentage of blue cells was obtained by counting cells with an inverted microscope as described in other sections.

In vitro Phagocytosis Measurements

The percentage of the cells obtained from the macrophage purification procedure that were capable of non-specific phagocytosis was measured by *in vitro* latex bead phagocytosis. The method used was similar to that reported by van Furth et al. (1980).

Once the testicular macrophage isolation procedure was completed, 3 ml of medium 199 containing 10% FBS and latex beads (3 µl of bead suspension/10 ml medium; bead suspension supplied as 10% w/v) was added to the wells. The plates were incubated (34°C, 5% CO_2) for 2 h. The cover slips were then removed, quickly air dried and fixed in methanol for 10 min. A Giemsa stain was used to improve the contrast under the microscope. The Giemsa stain was done by diluting the stock stain (obtained from the Pathology department) 1:10 in 0.1 M phosphate buffer and by submitting the cover slips to an ethyl alcohol series (90%, 80%, 70%) to hydrate the cells. The cover slips were then treated with acid alcohol (70% ethyl alcohol:glacial acetic acid, 100:1), washed under running water, rinsed with distilled water and immersed in the Giemsa stain solution for 30 min. Finally, the stained cover slips were washed with distilled water and air dried before counting the phagocytic cells using an inverted microscope as described in other sections. Results using this methods show that 80.6% to 84.5% of the cells attached to the cover slip are capable of non-immune phagocytosis.

Statistical Analysis

Several statistical tests were used to analyse the experimental results. In most cases the results from replicate experiments were averaged and statistical analysis performed on the combined data. However, the levels of androgen released by Leydig cell cultures can vary widely between preparations. Consequently, combining the results from several experiments often resulted in a very large standard error. In these cases, statistical analysis failed to detect differences between groups that were detectable in separate experiments. Two approaches were taken to circumvent this problem. In some cases, the data was standardized to represent the changes in androgen release in relation to the control group. Thus the data was expressed as "% of control" before results from separate experiments were combined. Comparison between treatment groups and the control group was done using a one-sample t-test, which compares a result with its standard error to a value which does not have a standard error. Where this method of standardizing the data is used, untransformed data is also provided in a separate figure. In addition, the results of statistical analysis on individual experiments are compared to that done on the normalized data. This insures that the data transformation did not bias the results. The second approach used to deal with the inter-experiment variability in androgen release was to describe statistical results on a 'per experiment' basis. This approach was used when the number of replicate experiments was low and the results were not always consistent between experiments.

Statistical analysis of untransformed data from combined results or for individual experiments was done using an analysis of variance (AOV) followed by the appropriate post-hoc test. Two-way AOVs were used to detect interactions between different treatments (eg. IL-1α and PTX) and one-way AOVs were used to detect differences between groups. When the experiment consisted of only two treatment groups, a two-tailed Student's t-test was used. This test is the equivalent to a one-way AOV. Post-hoc tests were chosen to match the type of comparison needed. A two-tailed Student's t-test was used when only a few comparisons were made. In a few cases, one-tailed t-tests were chosen but only when the direction of the changes induced by the treatment had already been established and the test only aimed at detecting significant changes. When multiple but predetermined comparisons were made, the Fisher's Product of Least Square Difference (PLSD) procedure was used. This statistical test was designed for that purpose (Steel & Torie, 1960). For comparisons of numerous treatment groups with the same control group, the two-sided Dunnett t-test procedure was used (Steel & Torie, 1960). A probability level of less than 0.05 was accepted as a significant difference between means.

RESULTS

In preliminary experiments, it was observed that treating Leydig cell cultures with 50 U IL-1α/ml, the half-maximal concentration required to stimulate PGE_2 production by human fibroblasts (Gubler et al., 1986), resulted in an increase in 24 h basal androgen release and in an inhibition of LH-stimulated androgen release. This was a novel finding that prompted a more systematic study of these effects.

To facilitate the interpretation of the experimental results, the typical changes in androgen release over time by adult rat Leydig cells in culture will be briefly described. The steroidogenic function of adult rat Leydig cells deteriorates quite rapidly in primary culture, although the cells themselves remain alive for quite some time (Murphy, 1983). The Introduction dealt with the possible cause of the loss of steroidogenenic function: evidence that a loss of 17α-hydroxylase function, caused either by free radical damage or by estrogen-mediated inhibition of its synthesis, is to blame. Why this occurs *in vitro* has not been established and no method of completely circumventing the problem exists. The use of antioxidant or low O_2 partial pressure in the incubator help reduce the time-related drop in androgen release but after a few days in culture, steroidogenesis still drops down to very low levels.

Changes in 24 h Basal Androgen Release Over Time in Untreated Leydig Cells in Culture

Figure 2 A shows the changes in 24 h basal androgen release over 7 consecutive days for untreated Leydig cell cultures. In our system, the average androgen production during the first 24 h of culture was approximately 18 ng per 100,000 cells. On day 2, there was 35% drop in production and the decline continued thereafter. On day 7, androgen release was less than 3% of that of day 1. The sharp decline that occurs between day 1 and day 2 is believed to be due, at least in part, to the loss of cells that had either attached poorly to the culture well bottom or were damaged in the isolation process and died during the first 24 h of culture while subsequent decline is more linked to steroidogenic enzyme damage (Murphy, 1983).

Changes in the Response of Untreated Leydig Cells to LH Over Time

The response of Leydig cells to LH also declines with increased culture time. Figure 2 B shows the amounts of androgen released by Leydig cells in response to a maximally effective concentration of LH (100 ng/ml) in a 4 h period following either 24 h or 96 h in culture. Basal androgen release during that same period is also shown. These results were obtained from the same cell preparation, that is the response to LH was measured, on separate wells, at 24 h and 96 h after the isolation of the cells. These experiments demonstrate that the response of Leydig cells to LH drops dramatically over time. In this

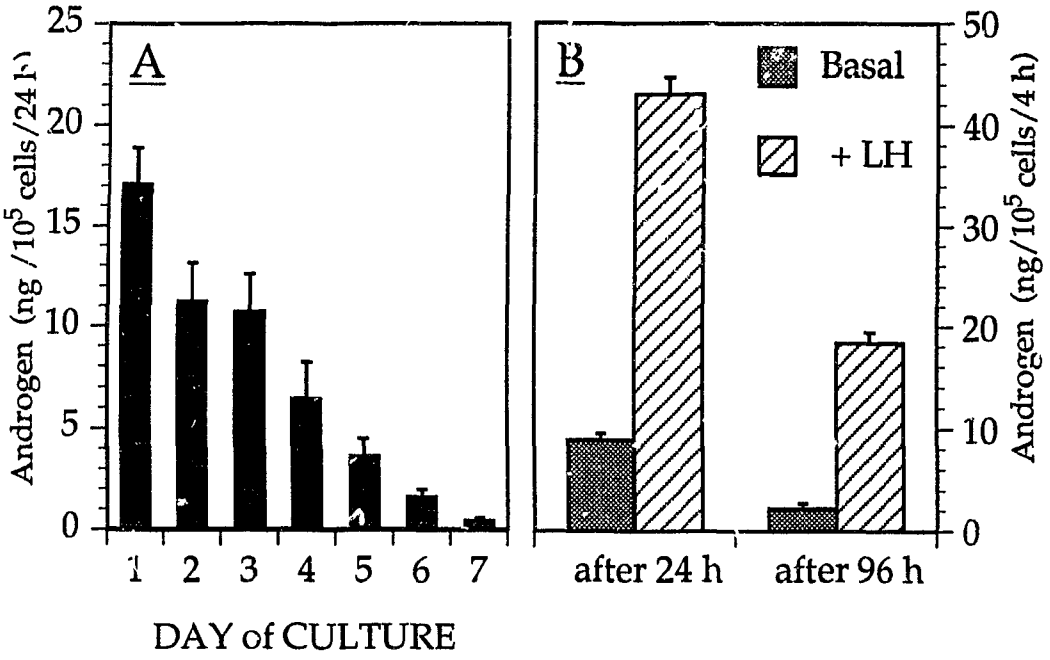

Figure 2.

Release of androgen by Leydig cell cultures. Panel A represent s the changes in basal androgen release during 7 consecutive 24 h periods. Values are the mean ± SEM of 25-26 (day 1-3), 7 (day 4), or 3-4 (day 5-7) separate experiments. Panel B illustrates the response of Leydig cell cultures to the stimulatory effect of a maximally effective concentration of LH (100 ng/ml) during a 4 h period after either 24 h or 96 h of culture. Values are from a representative experiment and are the mean ± SEM of 4 replicate wells per treatment group.

experiment there was a 57% decline in LH-stimulated androgen release between day 1 and day 4. In an identical experiment a 50% drop was observed. Basal androgen release in these two experiment had dropped by 75% and 65% during the same period of time. While the extent of the changes can vary between cell preparations, the response presented here is quite typical.

Effect of IL-1α on Androgen Release by Leydig Cells in Short Term Incubations.

Leydig cells were purified as described in Materials and Methods but instead of being cultured in multiwell plates, approximately 40,000 cells (in 0.5 ml medium 199) were dispensed into plastic culture tubes. IL-1α was added to half the tubes and LH (100 ng/ml) added to half of the Control tubes and half of the IL-1α treated. The air space in the tubes was flushed with 5% CO_2/95% air and rapidly capped. The tubes were then incubated in a shaking water bath (34°C) for 3 h. In these preliminary experiments concentrations of IL-1α ranged from 0.05 U/ml to 100 U/ml. There was no change in basal androgen release in any of the 4 experiments performed except where a small decrease in androgen release was seen with 2.5 U/ml in one case (AOV, $p<0.05$ and $p=0.02$ for Control vs 2.5 U IL-1/ml) and 0.5 U/ml in another experiment (AOV, $p<0.05$ and $p=0.0046$ for Control vs 0.5 U IL-1/ml). These changes were small (82% and 86% of Control, respectively) and inconsistent from experiment to experiment. Similarly IL-1α did not have any consistent effect on LH-

stimulated androgen release. Consequently, short term incubations were not further used to assess the role of IL-1α in Leydig cells steroidogenesis.

Dose Related Effect of IL-1α on 24 h Basal Androgen Release by Leydig Cells in Primary Cultures.

Leydig cells were prepared as previously described and cultured in the presence or absence of increasing concentration of IL-1α for 72 h. The medium (medium 199, supplemented as described in Materials and Methods) was removed every 24 h and replaced with fresh medium containing the appropriate amount of IL-1α. Figure 3 illustrates the release of androgen by the cells for each of the 3-24 h periods expressed as percent of Control. There was a significant effect of IL-1α on each day ($p<0.01$ by AOV, on data expressed as % of control and on androgen levels in each individual experiments). On day 1 of treatment the response was small (18-24%) but significant with the highest concentration of IL-1α (100 and 300 U IL-1α/ml, $p<0.01$ for all individual experiments by two-sided Dunnett t-test). On subsequent days, a significant response to as little as 30 U/ml ($p< 0.05$ for one experiment and $p<0.01$ for two experiments, by two-sided Dunnett t-test) was seen in all experiments. In addition, the magnitude of the response to the various doses of IL-1α increased with the duration of the culture, such that a maximally effective dose of IL-1α (100 U/ml) resulted in a 24.1% ± 5.85% increase above Control in 24 h androgen release on the day 1 of treatment but in a 141.5% ± 46.2% and

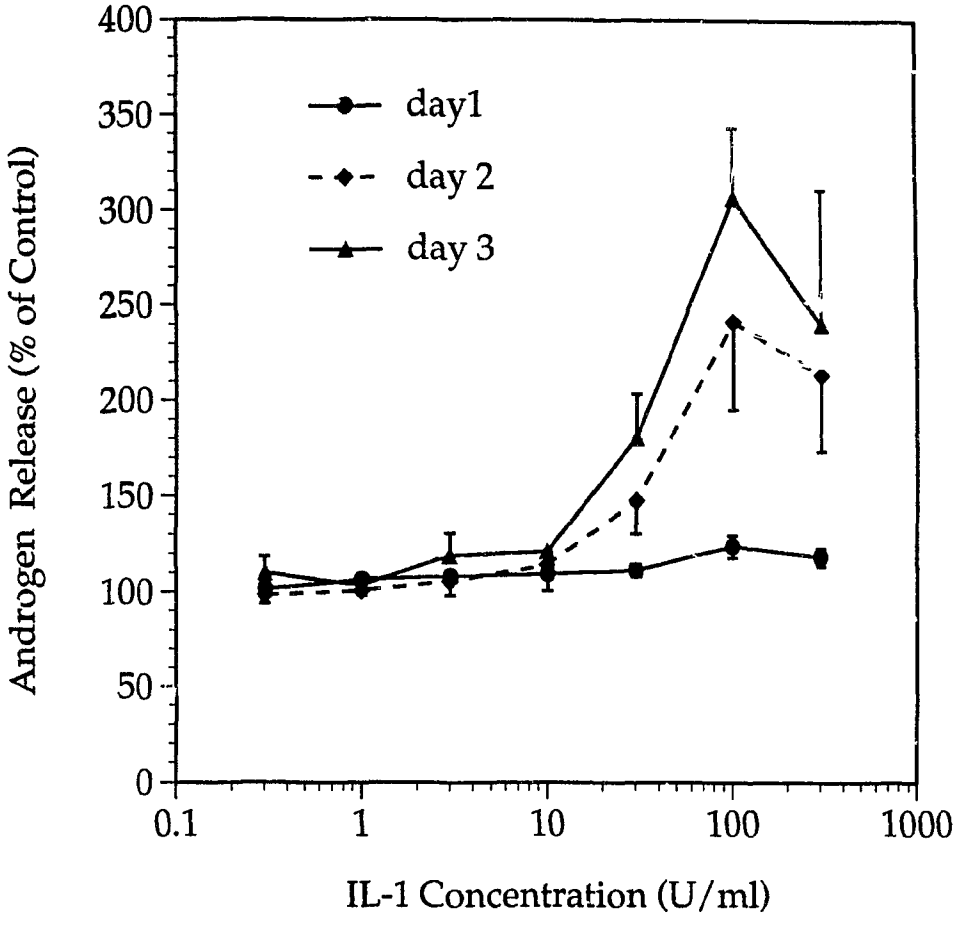

Figure 3.

Effect of time and concentration of IL-1 on basal androgen release. Cells were cultured in the presence of various concentration of IL-1 for 3 days. The medium was replaced every 24 h and fresh IL-1 added. Values are the mean of 2-3 separate experiments. Results are expressed as a percentage of androgen release by control (untreated) cells. The concentration of IL-1 had a significant stimulatory effect on each of the three days ($p < 0.01$ for each day by AOV), although the effect was more marked on days 2 and 3 of the experiment.

206% ± 36.5% increase above Control on the day 2 and 3, respectively. The half-maximal and maximal effective concentrations were 40-50 U/ml and 100 U/ml on day 2 and 3.

Effect of 100 U IL-1α/ml on 24 h Basal Androgen Release

The accumulation of androgen in the culture media over six consecutive 24 h periods in the presence or absence of 100 U IL-1/ml is shown in Figure 4. In the absence of IL-1α, androgen release by Leydig cell cultures decreased markedly between the day 1 and 2 of culture and then gradually to the end of the culture. In contrast, IL-1α-treated cells released significantly more androgen during the first 4 days of culture than control cells (two-tailed, paired t-test). The stimulatory effect was still marginally significant (p=0.062, n=4) on day 5 but was not significant on day 6. The maximum stimulatory effect was reached on day 3 of culture where IL-1α-treated cultures release 2.71 times more androgen than control cultures.

Effect of Delaying the IL-1α Treatment on the Time-related Changes in 24 h Basal Androgen Release

As previously stated, the decrease in androgen production seen after the first change of media is often attributed to the loss of cells that have either died or failed to attach to the culture dish during the initial culture period. In order to test the possibility that IL-1α may be exerting its stimulatory effect on basal

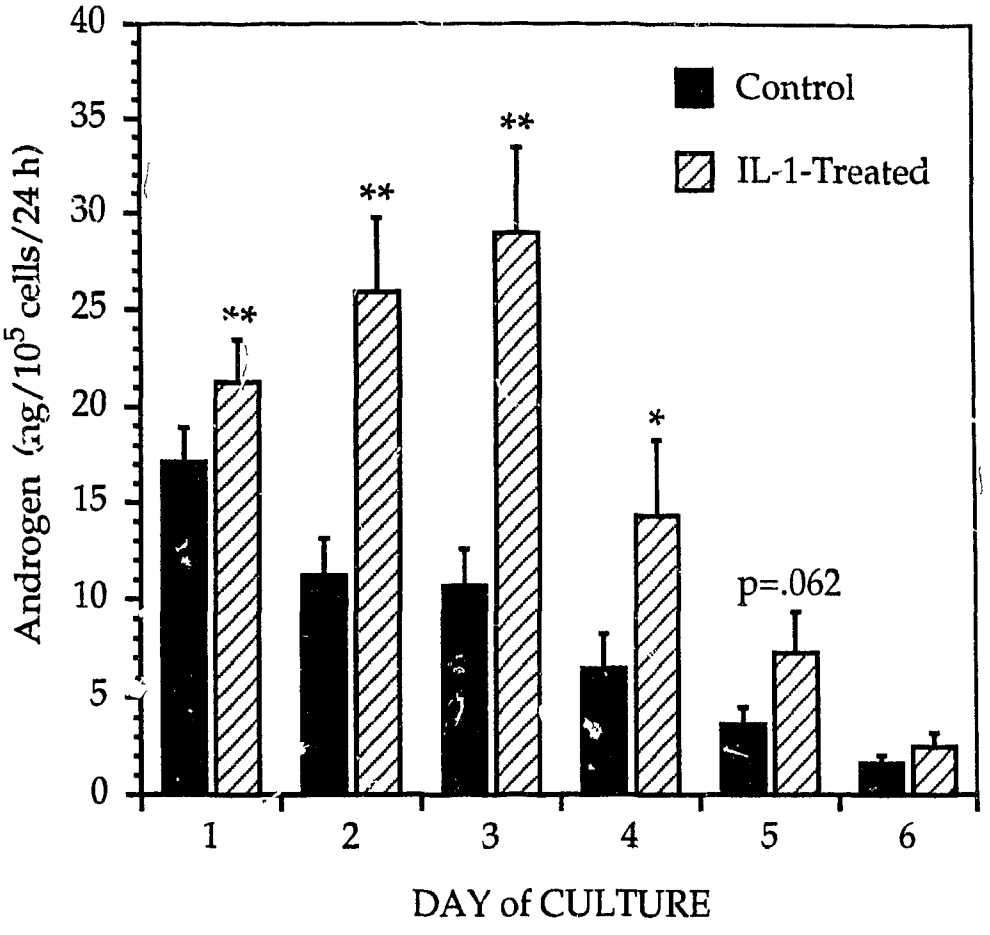

Figure 4.

Effect of IL-1 on 24 h basal androgen release over 6 consecutive days. Cells were cultured in the presence or absence of IL-1 (100 U/ml) for 6 days. The medium was replaced every 24h and fresh IL-1 added. Values are the mean ± SEM of 25-26 (day 1-3), 7 (day 4) and 4 (day 5-6) separate experiments. Statistical analysis consisted of paired, two-tailed t-tests between the response by control and IL-1 treated cells on the same day. *, $p<0.05$; **, $p<0.01$.

androgen release by either 'rescuing' dying cells or by helping their anchoring, the introduction of IL-1α in the culture media was delayed by 24 h or 48 h. Such delay allowed such cell loss to take place before IL-1α was added. These experiments (Figure 5) showed that delaying the addition of IL-1α did not eliminate the stimulatory effect of IL-1α. The maximum stimulatory effect of IL-1α was reached on the third day of IL-1α treatment and was similar in magnitude (242% - 277% of control), whether IL-1α was present from the start of culture (A) or its addition was delayed by 24 h (B) or 48 h (C). Similar levels of androgen were released by these cultures; in the one experiment where IL-1α was introduced at 0 h, 24 h and 48h, basal androgen levels on the third day in the presence of IL-α were 9.06 ± .42, 16.78 ± .12 and 14.74 ± .66 ng/ml for the three treatment group, respectively. In other experiments, the DNA content of the cultures was assessed at the end of 3 days of culture in the presence of absence of IL-1α and was unaffected by IL-1α (Table 4.). These results indicated that changes in cell number could not account for the changes in androgen release seen in IL-1α-treated cells.

Reversibility of the Effect of IL-1α on Basal and LH- stimulated Androgen Release

The reversibility of the stimulatory effect of IL-1α on 24 h basal androgen release was evaluated by culturing Leydig cells in the absence of IL-1α for 4 days (control) or in the presence of 100 U IL-1α/ml during either

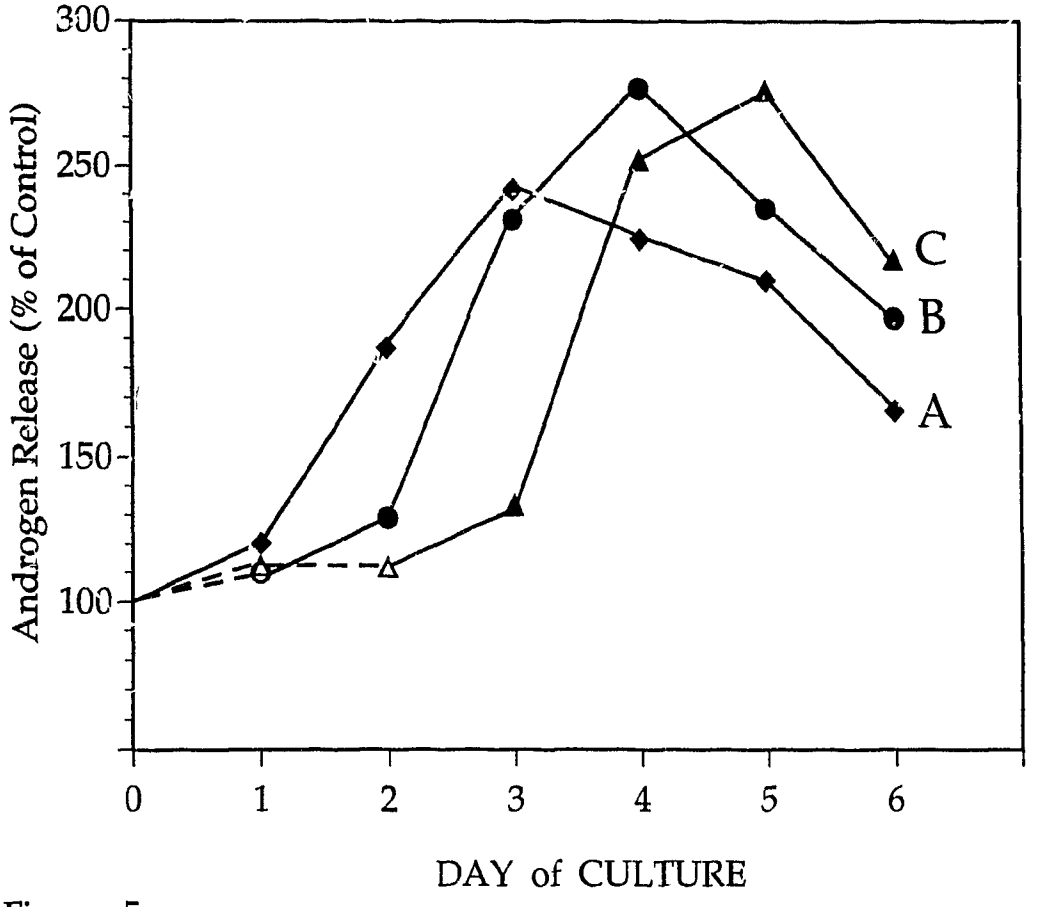

Figure 5.

Effect of delaying the addition of IL-1 to the culture on basal androgen release. Cells were cultured for up to six days. Group A (◆) was cultured in the presence of IL-1 (100 U/ml) from the time of plating onwards. Group B (●O) was cultured for 24 h in control medium and in the presence of IL-1 thereafter. In group C (▲Δ), IL-1 was introduced in the culture medium after 48 h of culture in control medium. The medium was collected every 24 h and replaced with fresh medium containing the appropriate treatment. Open symbols (OΔ) preceded by a broken line indicate a period of culture in control medium and filled symbols (◆●▲) preceded by a solid line indicate the presence of IL-1 in the medium during that period. Values are the mean of 2-3 experiments. Error bars were omitted for clarity of the presentation.

DNA Content in µg/well

Control	IL-1	NDGA	NDGA + IL-1
1.307 ± .148	1.280 ± .152	1.315 ± .282	1.203 ± .291

Table 4.

Effect of IL-1 and/or NDGA on the DNA content of Leydig cell cultures. Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml and/or 10 µM NDGA. At the end of the experiments, the culture medium was collected and the DNA content of the cultures assessed. Values are the mean ± SEM of 2-3 separate experiments with 6 replicate wells per experiment. There was no difference between groups by AOV.

the first day of the 4 day culture only (group 'a') or during the 4 days of culture (group 'b'). Results are shown in Figure 6 A. Wells in which IL-1α treatment was discontinued were washed with 1 ml of supplemented medium to reduce the amount of IL-1α that might remain in the medium but no other procedure was used to further reduce residual IL-1α in the well or on the cells.

As expected there was a small but significant stimulatory effect of IL-1α during the first day of culture ($p<0.05$ for both group 'a' and group 'b'). However, on day 2 of culture, cells from group 'a' released significantly more androgen ($p< 0.05$) than control cells, despite the absence of IL-1α in the culture media. In fact, the level of stimulation was not different from that observed for cells continuously exposed to IL-1α (group 'b'). On the second day without IL-1α, androgen release in group 'a' was still increased compared to untreated cells but was lower than in group 'b', the cultures treated continuously with IL-1α (but not significantly lower by t-test). By the third day without IL-1α, the effect of the 1-day IL-1α treatment (group 'a') was completely reversed. In a similar experiment (data not shown) in which cells were treated with IL-1α during the first 48 h of culture and then discontinued, the stimulatory effect of IL-1α was also found to be maintained fully or in part during the two 24 h periods following removal of IL-1α but to be completely reversed on the third day of culture without IL-1α treatment.

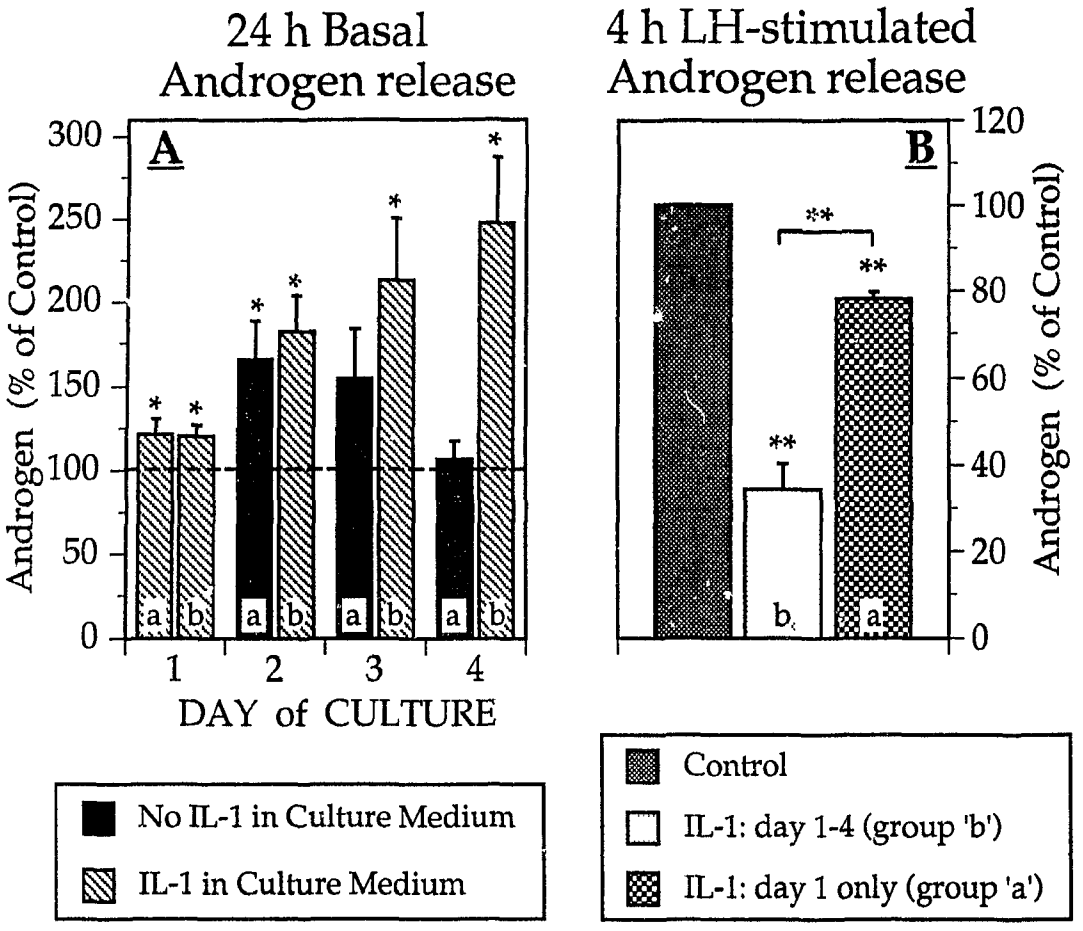

Figure 6.

Reversibility of the effects of IL-1 on basal and LH-stimulated androgen release. Cells from group '**a**' were cultured in the presence of IL-1 (100 U/ml) for 24 h and then cultured for an additional 3 days in control medium. Cells from group '**b**' were cultured in the presence of IL-1 for 4 days. The control group was cultured without IL-1. Basal androgen release was measured each day (panel **A**). At the end of the 4 days, the response to the stimulatory effect of a maximally effective concentration of LH (100 ng/ml) was measured for 4 h (panel **B**). Values are the mean ± SEM of 3-4 experiments expressed as '% of Control'. *, $p<0.05$; **, $p<0.01$ compared to control by one-tailed, one-sample t-test or, where indicated (—), between treatment groups by two-tailed t-test.

The response of cells to LH was also measured. As seen in Figure 6 B, cells cultured in the presence of IL-1α for 4 days (group 'b') released only 34% of the androgen released by control cells in response to LH ($p<0.01$ by one-tailed, one-sample t-test). Cells from group 'a', those treated with IL-1α during the first 24 h of culture only, also released significantly less androgen than control cells in response to LH (78% of control, $p<0.01$ by one-tailed, one-sample t-test) but significantly more than cells from group 'b' ($p<0.01$ by two-tailed t-test).

Dose Related Effect of IL-1α on LH-stimulated Androgen Release

The inhibitory effect of IL-1α on LH-stimulated androgen release was concentration-dependent. Results obtained by treating Leydig cells cultures with 0.3 U IL-1α/ml to 300 U IL-1α/ml for 3 days followed by a 4 h stimulation with a maximally effective concentration of LH (100 ng/ml) are shown in Figure 7. Within the range of IL-1α concentrations used for these experiments, a maximally effective concentration of IL-1α could not be identified. The inhibitory effect of 30 U IL-1α/ml was significant in two out of three experiments. Since 100 U IL-1α/ml had a significant inhibitory action in all experiments ($p<0.05$ by two-sided Dunnett t-test) and since this concentration was maximally effective at stimulating 24 h basal androgen release (see Figure 3) all subsequent experiments were performed using 100 U IL-1α/ml.

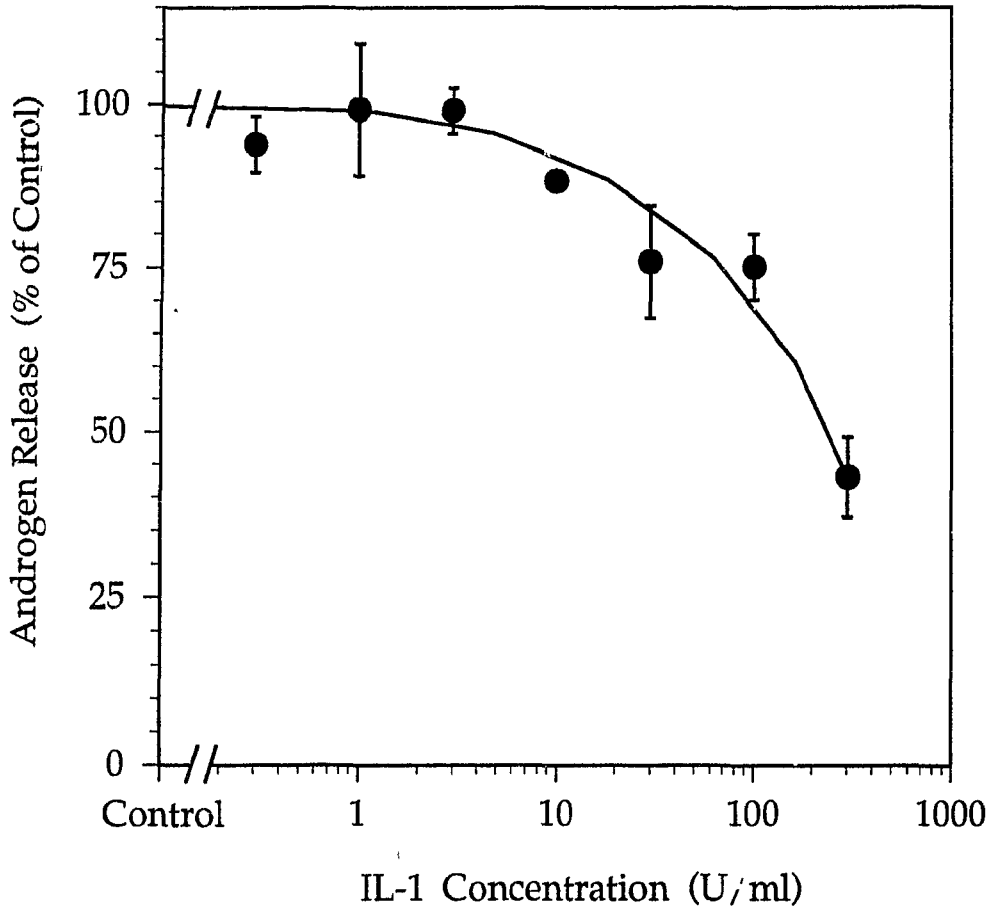

Figure 7.

Effect of concentration of IL-1 on LH-stimulated androgen release. Cells were cultured for 3 days in the presence or absence of various concentrations of IL-1. The medium was replaced every 24 h and fresh IL-1 added. At the end of the 3 day culture period the medium was changed once more, IL-1 and/or 100 ng LH/ml added where appropriate and the cells cultured for an additional 4 h. Basal androgen release was also measured. Values are the mean ± SEM of 2-3 experiments. Statistical analysis was done on individual experiments and is described in the text.

Effect of 100 U IL-1α/ml on LH-stimulated Androgen Release

At the end of either 24 h, 48 h or 72 h of culture in the presence or absence of IL-1α (100 U/ml), Leydig cells were further cultured for a period of 4 h in the presence or absence of a maximally effective concentration of LH (100 ng/ml). There was no significant difference in LH-induced androgen release between control and IL-1α-treated cells after 24 h or 48 h of culture (Figure 8). After 72 h in the presence of IL-1α (Figure 9) there was a significant inhibition of LH-stimulated androgen release compared with control cells (72% of control; $p < 0.01$ by paired two-tailed t-test). Basal androgen release was significantly elevated in all separate experiments ($p < 0.05$) even though the difference did not reach significance for the combined results for the '48 h in culture' experiment.

Effect of 100 U IL-1α/ml on the Dose-related Response of Leydig Cells to LH

The response of Leydig cells to increasing concentrations of LH after 72 h of culture in the presence or absence of IL-1α was measured. The resulting dose-response curve to LH (Figure 10 A) revealed an apparent biphasic effect of IL-1α on LH-stimulated androgen release. At low concentrations of LH (less than 1 ng LH/ml), IL-1α-treated cells released more androgen than control cells in all experiments ($p<0.05$ by Fisher's PLSD but not significant for combined results). For LH concentrations greater than 1 ng/ml, IL-1α treatment significantly inhibited androgen release (for 1 ng LH/ml; $p<0.01$ by

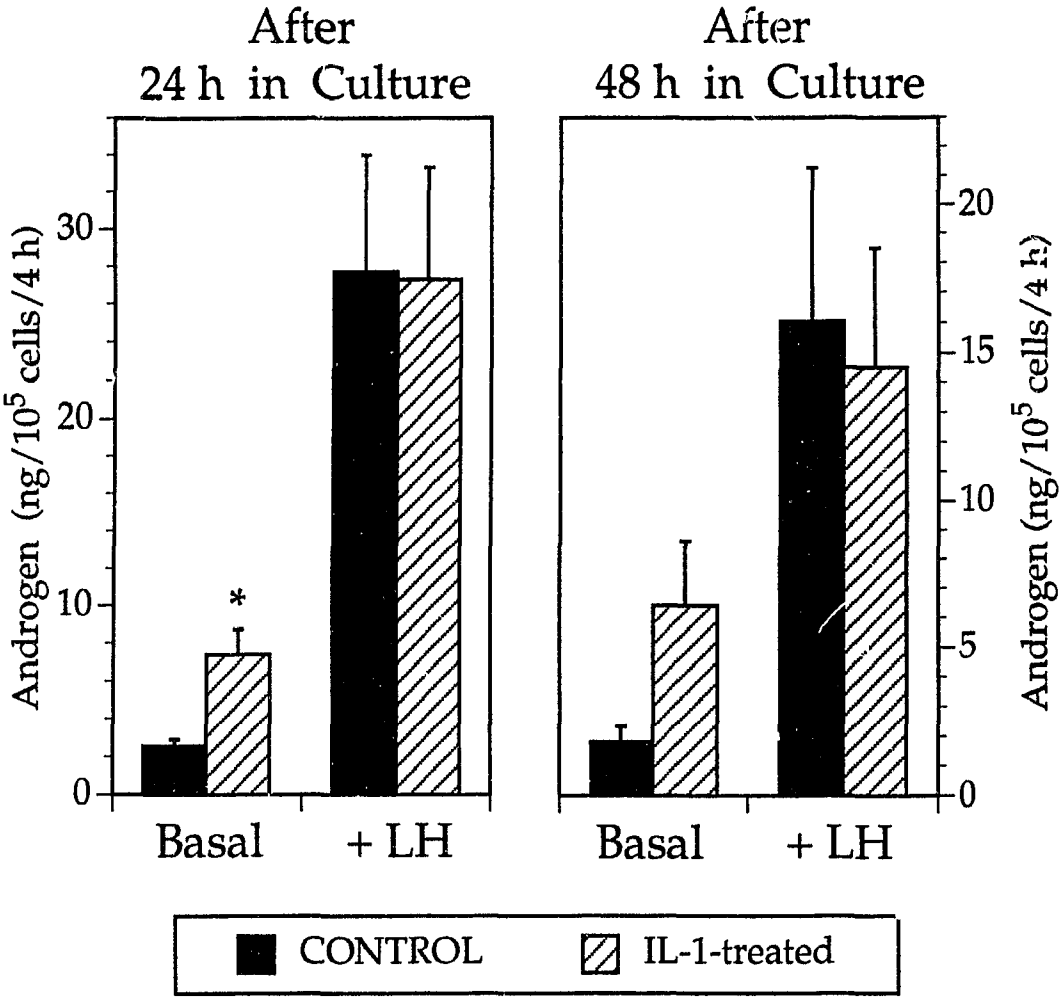

Figure 8.

Effect of 24 h or 48 h of culture with IL-1 on LH-stimulated androgen release. Cells were cultured in the presence or absence of IL-1 (100 U/ml) for either 24 h or 48 h. The medium was replaced daily and fresh IL-1 re-added. At the end of the culture period, cells were stimulated with 100 ng LH/ml for a 4 h period. Values are the mean ± SEM of 3 (48 h culture) or 4 (24 h culture) separate experiments. Basal androgen release was significantly increased by IL-1 treatment in all separate experiments. There were no significant changes in the response to LH in the presence of IL-1 by two-tailed t-test.

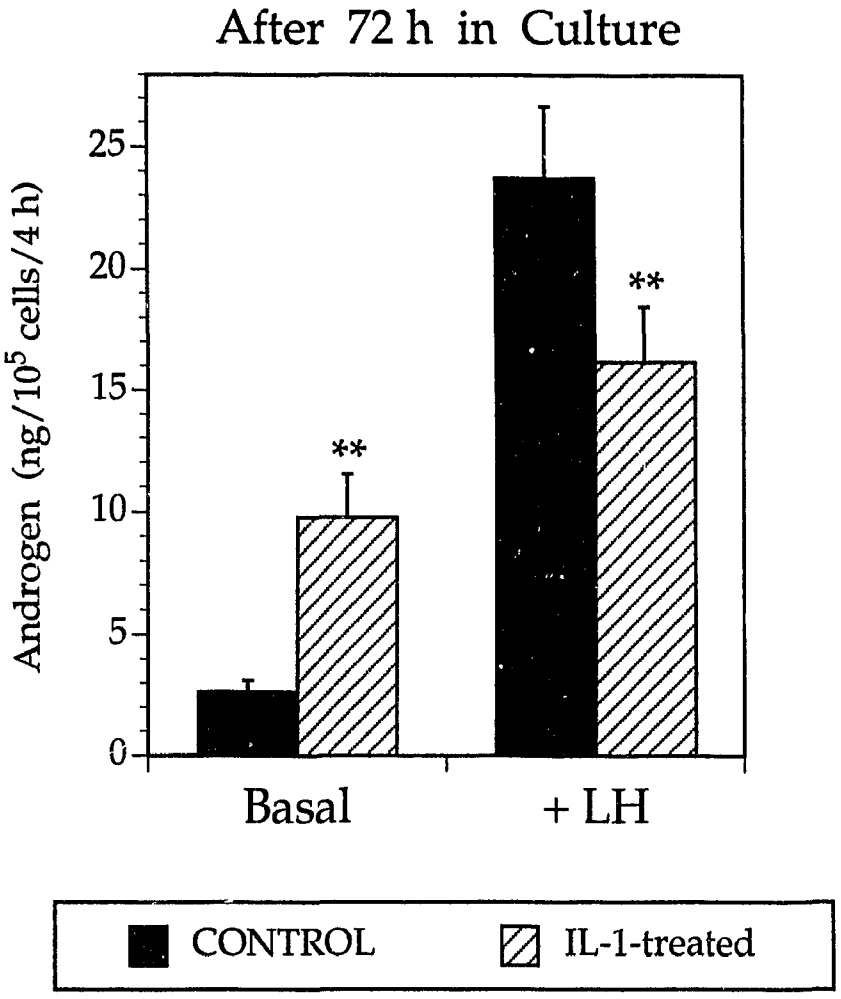

Figure 9.

Effect of 72 h of culture in the presence of IL-1 on LH-stimulated androgen release. Cells were cultured in the presence or absence of 100 U IL-1/ml for 72 h. The medium was changed every 24 h and fresh IL-1 added. At the end of the culture period the cells were stimulated with a maximally effective concentration of LH for a 4 h period. Values are the mean ± SEM of 27-29 separate experiments. **, $p<0.01$ by paired, two-tailed t-test.

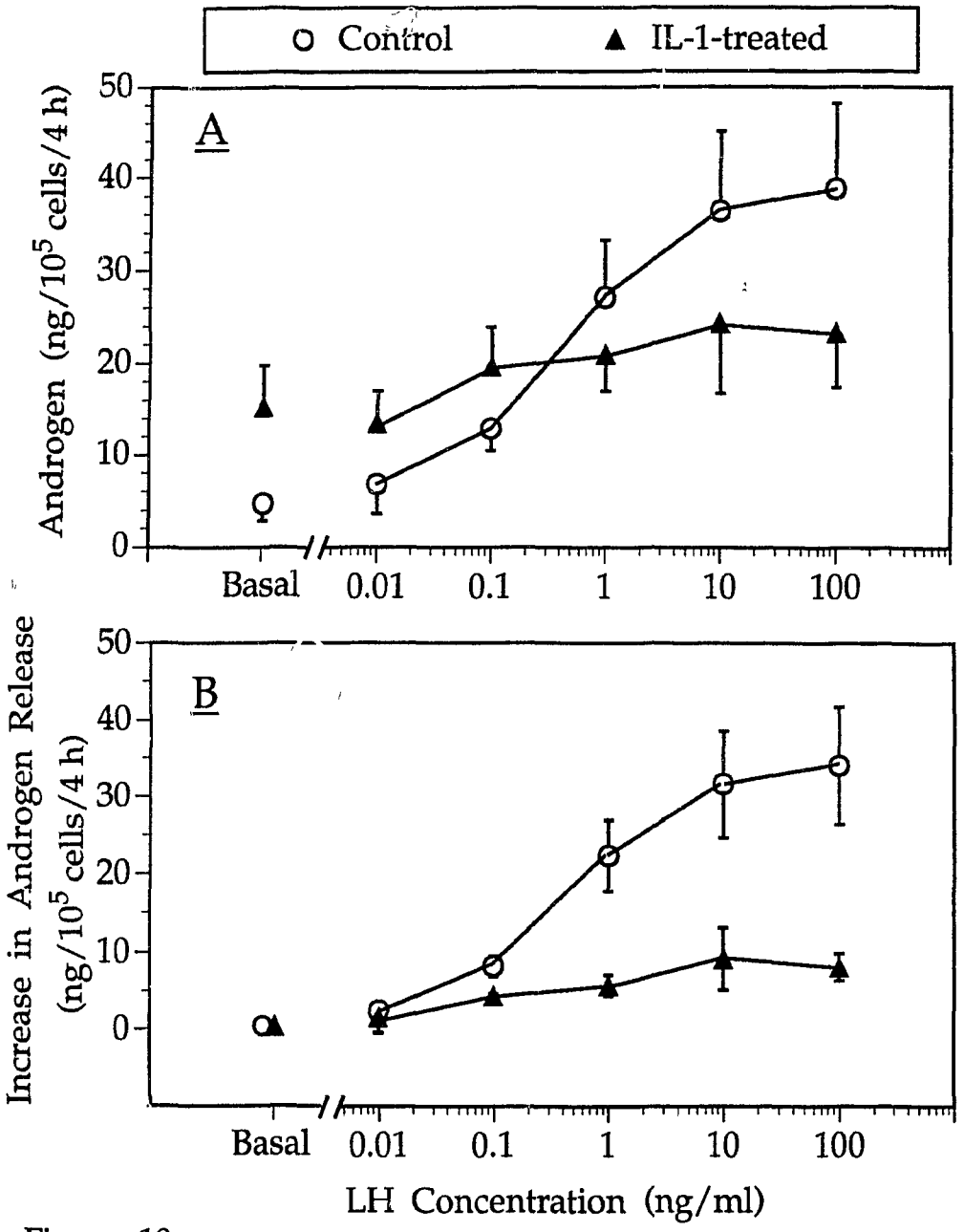

Figure 10.

Effect of IL-1 on the response of Leydig cells to various concentrations of LH. Panel A represents the response as androgen release in the medium while panel B represents the increase in androgen release over basal androgen levels. Cells were cultured in the presence or absence of IL-1 (100 U/ml) for 3 days and then exposed to various concentrations of LH for a period of 4 h. Values are the mean of 2-3 separate experiments. Statistical analysis was done on individual experiments and is described in the text.

Fisher's PLSD on 2 out of 3 experiments and $p<0.01$ for combined data). There was a large difference in basal androgen released by control and IL-1α-treated cells and statistical analysis of the data revealed a significant interaction ($p< 0.01$ for all experiments) between IL-1α treatment and LH concentration. To clarify the nature of the effect of IL-1 on the response of Leydig cells to LH the data were transformed to present the increase in androgen release induced by LH relative to the basal androgen levels in the corresponding treatment group (Figure 10 B). This transformation of the data revealed that IL-1α reduced the increase in androgen release at all concentrations of LH above 0.1 ng LH/ml ($p<0.01$ by Fisher's PLSD between Control and IL-1α-treated cells in 2 out of 3 experiments for 0.1 ng LH/ml and for all experiments for 1 to 100 ng LH/ml). Therefore, despite having a stimulatory effect on basal androgen release, IL-1α blunted the response of Leydig cells to the stimulatory effect of LH. In the presence of IL-1α, the EC_{50} for LH was 0.128 - 0.129 ng/ml while control EC_{50} was 0.455 - 0.497 ng/ml (calculated from combined results expressed both as androgen levels in the medium and as increase over basal).

Effect of IL-1β and IL-1α from a Different Supplier on Basal and LH-stimulated Androgen Release.

The effect of IL-1α obtained from a different supplier (Collaborative Research, C R) was compared to the effect of IL-1α from Hoffman-La Roche. Because of a limited supply of IL-1α from C R, only 40 U IL-1α/ml were used.

The results from one of two similar experiments are shown in Table 5. IL-1α from both sources induced similar effect on 24 h basal and 4 h LH-stimulated androgen release. Both preparations had a significant ($p<0.01$ or $p<0.05$ by two-tailed t-test) stimulatory effect on basal androgen release and a significant ($P<0.01$) inhibitory effect on the response to LH.

IL-1β had also induced an increase in 24 h basal androgen release but this type of IL-1 appears to be much more active than IL-1α. As seen in Table 6, 20 U IL-1β induced a marked increase of basal androgen release on day 1 and a 7-fold increase in basal androgen release on day 3. The increase induced by 100 U IL-1α/ml on day 1 is usually small (about 25% on average) and on day 3 of treatment the stimulatory effect is usually around 3-fold. The inhibitory effect of IL-1β was small and not significant. However, when the response to LH was expressed as the increase over basal, IL-1β had a significant inhibitory effect. It has been observed with IL-1α that a very large increase in basal androgen release is often accompanied by a smaller inhibition of the response to LH.

Site of Action for the Inhibitory Effect of IL-1α on Androgen Release

To determine the site of the inhibitory effect of IL-1α on LH-stimulated steroidogenesis, Leydig cell cultures were treated for 4 h with various concentrations of dbcAMP or pregnenolone after a 3-day culture period in the

Androgen Release (ng/100,000 cells)

	Control	IL-1alpha (from H-L)	IL-1alpha (from C R)
Day 1	18.09 ± .88	21.94 ± .32 **	21.53 ± .52 *
Day 2	7.35 ± .03	15.22 ± .76 **	13.97 ± .52 **
Day 3	5.33 ± .08	12.38 ± .75 **	10.56 ± .54 **
4 h LH-stimulated	24.76 ± .96	21.61 ± .96 p=0.059	20.25 ± .50 **

Table 5.

Comparison of the effect of IL-1a from two different suppliers. Cells were cultured in the presence or absence of 40 U/ml IL-1α obtained either from Hoffman-La Roche (H-L) or from Collaborative Research (C R). Four hour basal androgen release could not be measured because of the limited supply of IL-1α from C R. Values are the mean ± SEM of 4 replicate wells. *, $p<0.05$; **, $p<0.01$ by two-tailed t-test between IL-1-treated cells and control. There was no significant difference between cells treated with IL-1α from H-L and from C R.

Androgen release (ng/100,000 cells)

	Control	IL-1 beta (20 U/ml)
Day 1	16.41 ± .42	26.76 ± .48 **
Day 2	7.44 ± .14	38.64 ± 1.32**
Day 3	7.67 ± .16	48.47 ± 1.38**
4 h Basal	3.02 ± .68	23.66 ± .87 **
4 h LH	39.03 ± 4.03	37.58 ± 2.01
Increase over Basal	36.00 ± 3.53	14.17 ± 1.61**

Table 6.

Effect of IL-1β on androgen release by Leydig cells in culture. Cells were cultured as previously described in the presence or absence of IL-1β (20 U/ml) for 3 days. An additional 4 h incubation was done at the end of the culture period in the presence or absence of LH (100 ng/ml). Values are the mean ± SEM of 8 (24 h basal) or 4 (4 h basal and LH-stimulated release) replicate wells. **, $p<0.01$ by two-tailed t-test.

presence or absence of IL-1α (100 U/ml). Figure 11 illustrates tne androgen production induced by a submaximal (30 μmol/L) and maximal (300 μmol/L) concentrations of dbcAMP in control and IL-1α-treated cultures in a representative experiment. As expected, IL-1α-treated cultures released significantly more androgen under basal conditions than control cells ($p<0.01$). The response to 30 μM dbcAMP was slightly but significantly increased in IL-1α-treated cells ($p<0.01$). This effect was observed in the other 2 similar experiments ($p<0.01$ and $p=0.067$). When the response was expressed as the increase in androgen release over basal levels, a significant inhibitory effect of IL-1α was observed in 2 out of 3 experiments ($p<0.01$, $p<0.05$ and $p=.12$). With a higher concentration of dbcAMP (300 μM), IL-1α-treated cells released less androgen than control in 2 out of 3 experiments ($p<0.01$ for two experiments and $p=.29$ for one experiment) and the increase androgen release over basal was significantly smaller in IL-1α-treated cultures in all 3 experiments ($p<0.01$). IL-1α had similar effects on the response to 0.3 μM pregnenolone and 3 μM pregnenolone. The results from a representative experiment are shown in Figure 12. The response to the lower concentration of pregnenolone was somewhat variable, ranging from a slightly stimulatory effect ($p<0.01$ in one experiment) to the absence of significant change in androgen release ($p=.78$ and $p=.14$). IL-1α had an inhibitory effect on the increase over basal but this effect was not always significant with 0.3 μM pregnenolone. The results were more consistent when the higher concentration of pregnenolone was used (3 μM).

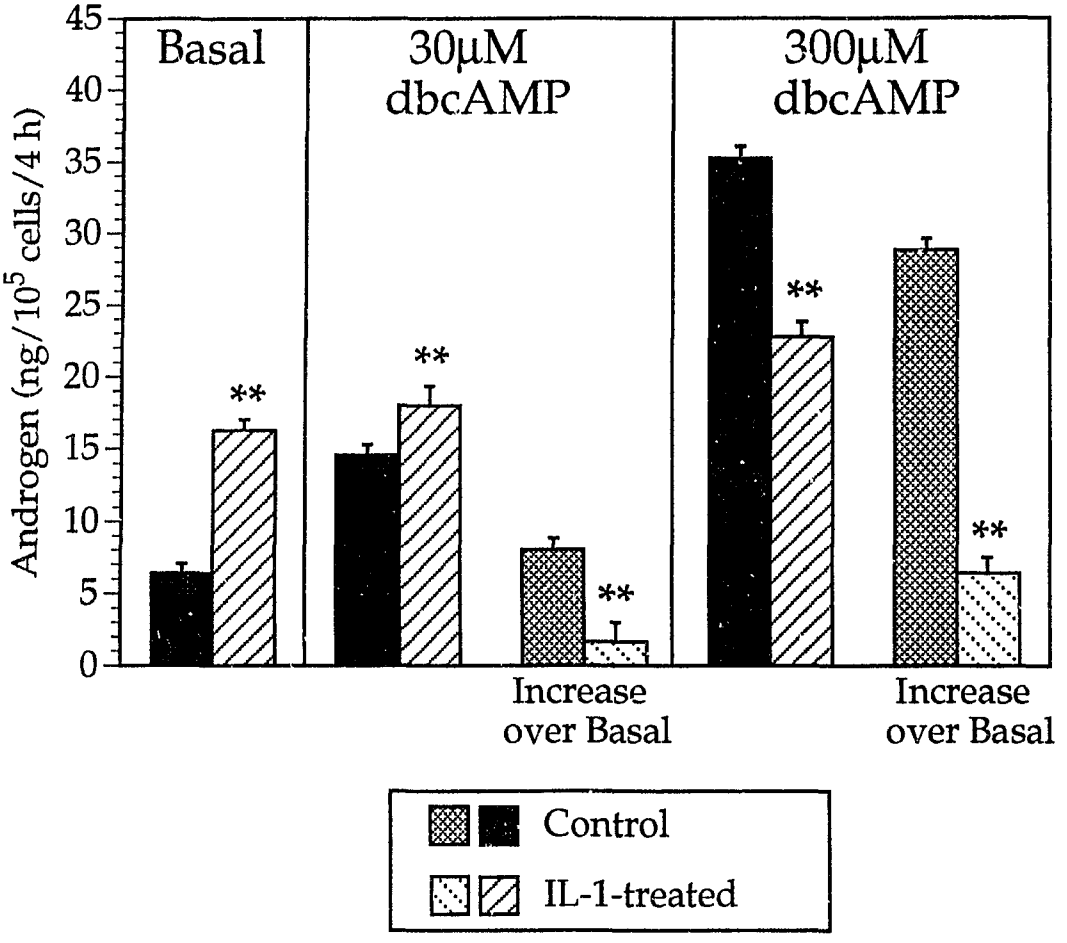

Figure 11.

Effect of IL-1 on the response of Leydig cells to various concentrations of dbcAMP. Cells were cultured for 3 days in the presence or absence of IL-1 (100 U/ml) and then exposed to 30 μM or 300 μM dbcAMP for 4 h. The figure shows the effect of IL-1 under basal conditions and the response to dbcAMP expressed as androgen release and as the increase in androgen release over basal levels. **, $p<0.01$ by two-tailed t-test for one of three similar experiments.

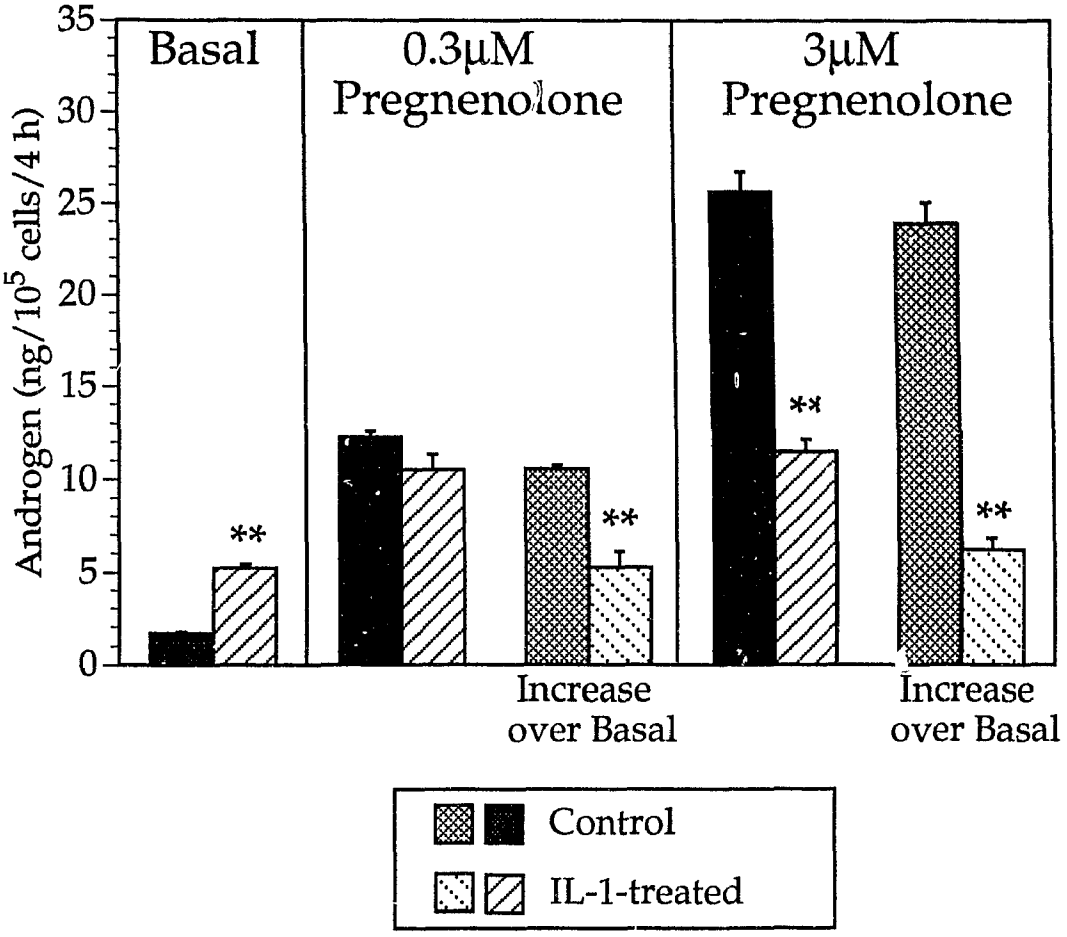

Figure 12.

Effect of IL-1 on the response of Leydig cells to various concentrations of pregnenolone. Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml and then exposed to 0.3 μM or 3 μM pregnenolone for 4 h. The figure shows the effect of IL-1 under basal conditions, and the response to pregnenolone expressed as androgen release and as increase in androgen release over basal levels. **, $p<0.01$ by two-tailed t-test for one of three similar experiments.

There was a significant inhibition of androgen release ($p<0.01$) and of the increase over basal ($p<0.01$) in all 4 experiments. Similar results were obtained with 25-OH-cholesterol where only a small decrease in the increase androgen release over basal ($p=0.3$ and $p=0.098$) was seen with 1 μM 25-OH-cholesterol and a significant decrease was seen with 10 μM cholesterol (42.1% ± 4.6%, $p<0.05$). The data from these experiments indicate that IL-1α acts, at least in part, at a site distal to cAMP production and cholesterol side-chain cleavage.

Effect of IL-1α on cAMP Production

Several reports indicate that IL-1 may exert some of its effects via modulation of cAMP levels (Chedid et al., 1989; Shirakawa et al., 1988). Consequently, we measured the changes in cAMP levels in control and IL-1α-treated cells under basal and LH-stimulated conditions. Our results indicate that changes in cAMP levels did not always go hand in hand with changes in androgen release. There was no difference in basal cAMP and androgen levels during the first 4 h of culture in the presence of IL-1α (right panels on Figure 13 A and 13 B). However, after 48 h of culture basal cAMP levels were still not altered by IL-1α in contrast with basal androgen levels which were significantly increased ($p<0.01$) (left panels on Figure 13 A and 13B). As seen in Figure 14 A, LH-stimulated cAMP levels were unchanged during the first 4 h with IL-1α but were significantly inhibited after 48 h with IL-1α. The inhibition of cAMP levels was not accompanied by any changes in androgen release (Figure 14 B).

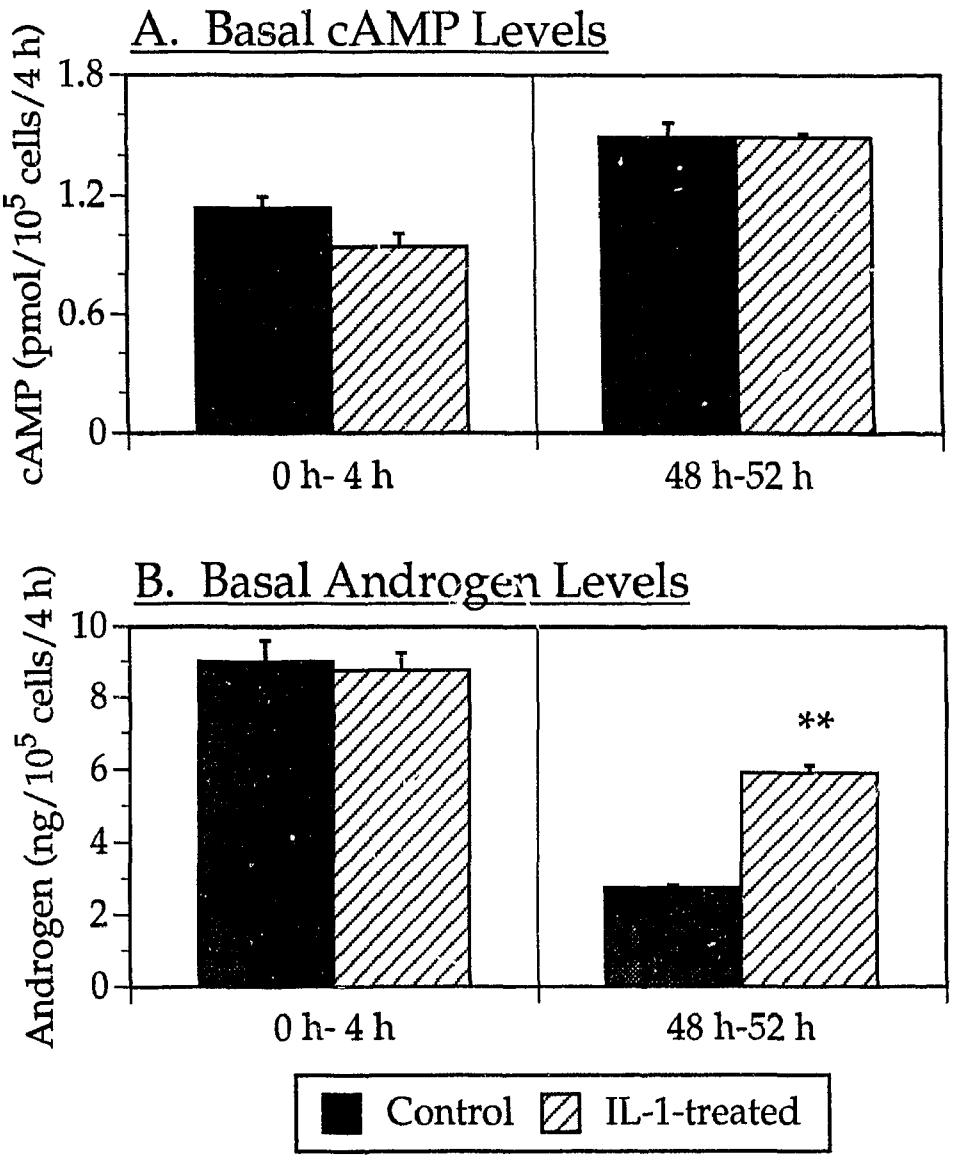

Figure 13.

Effect of IL-1 on basal cAMP and corresponding androgen levels. Levels of cAMP (A) and androgen (B) were measured during the first 4 h of IL-1 treatment (100 U/ml) or for 4 h after 48 h in the presence or absence of IL-1. Values are the mean ± SEM from 1 of 2 experiments for the '4 h with IL-1' data and from 1 experiment for the '48 h with IL-1' data. **, $p<0.01$ by two-tailed t-test.

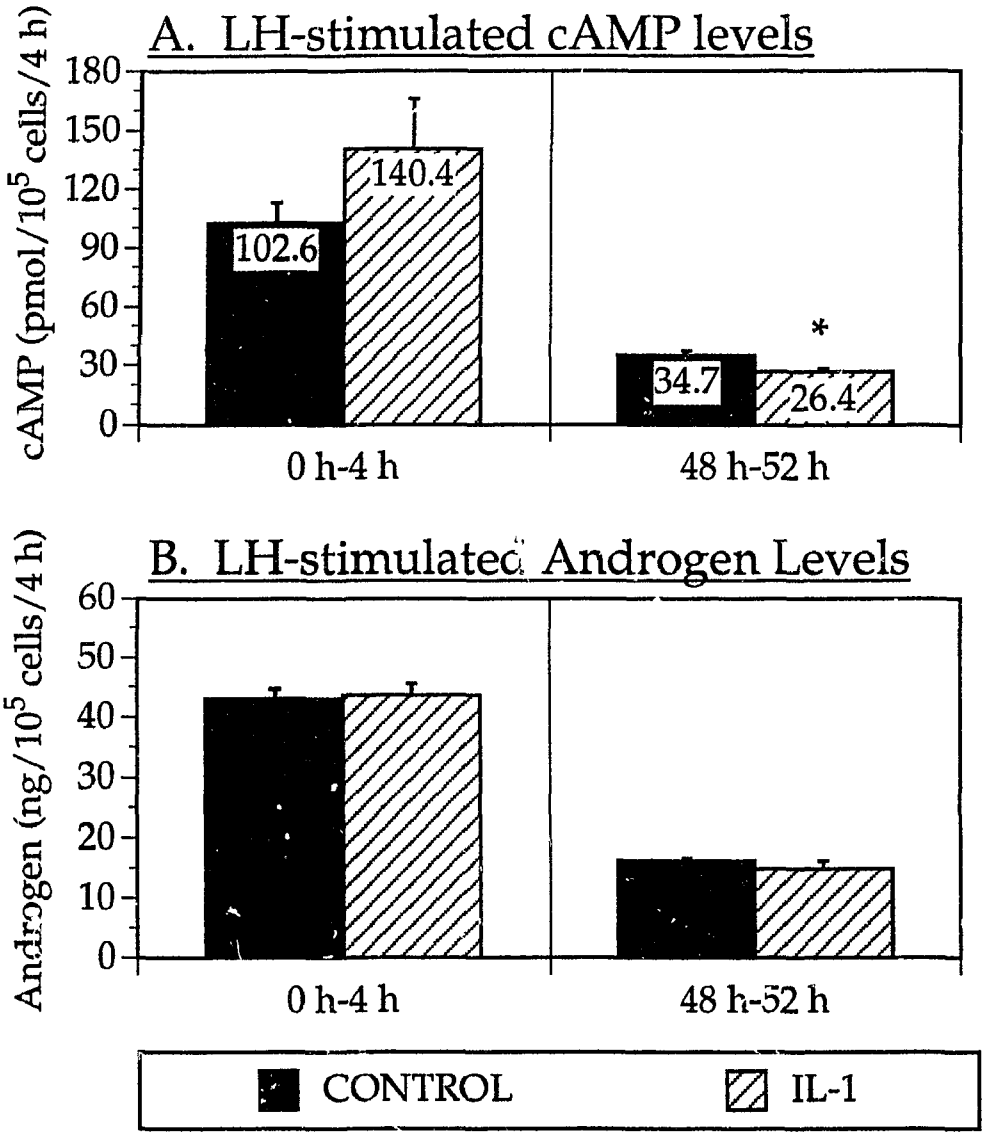

Figure 14.

Effect of IL-1 on LH-stimulated cAMP and corresponding androgen levels. LH-stimulated (100 ng/ml) cAMP (A) and androgen (B) levels were measured during the first 4 h of IL-1 treatment (100 U/ml) or for 4 h after 48 h in the presence or absence of IL-1. Values are the mean ± SEM from 1 of 2 experiments for the '4 h with IL-1' data and from 1 experiment for the '48 h with IL-1' data. *, $p<0.05$ by two-tailed t-test.

The effect of IL-1α on cAMP and androgen levels after 3 days of culture are summarized in Figure 15 which shows the results as % of Control. Actual levels of androgen and cAMP are shown in Figure 16-17. Statistical analysis was performed on the data expressed as % of control (by two-tailed, one sample t-tests) and on untransformed data (by two-tailed t-tests). Basal cAMP was significantly increased (211% ± 25.4% of control, $p<0.01$ and $p=0.07$ for untransformed data) in IL-1α-treated cells. Corresponding basal androgen levels were also increased (244.7% ± 40.8%, $p<0.05$ and $p<0.05$ for untransformed data). The release of cAMP (after 72 h) in the presence of a maximally effective concentration of LH (Figure 15 and Figure 18) was markedly reduced in IL-1α-treated cells, being only 36.9% ± 6.9% of the levels seen in control cells ($p<0.01$ and $p<0.01$ for untransformed data). Androgen release was also significantly inhibited (62.1% ± 6.9%, $p<0.01$). When a submaximally effective concentration of LH was used (1 ng/ml) (Figure 17 for untransformed data) a significant, or nearly significant increase in cAMP levels was seen ($p<0.01$ for 2 experiments and $p=0.068$ for the third experiment; for combined experiments, cAMP levels were 174.8% ± 27% of control, and $p<0.05$ for untransformed combined data). Androgen levels were, in contrast, inhibited in 2 out of the 3 experiments ($p<0.05$) and unchanged in the third. The small effect of IL-1α on the response to 1 ng LH/ml is in agreement with the results from the dose-response to LH experiments (see Figure 10 A). This concentration is close to the point where the effect of IL-1α shifts from being stimulatory to becoming inhibitory.

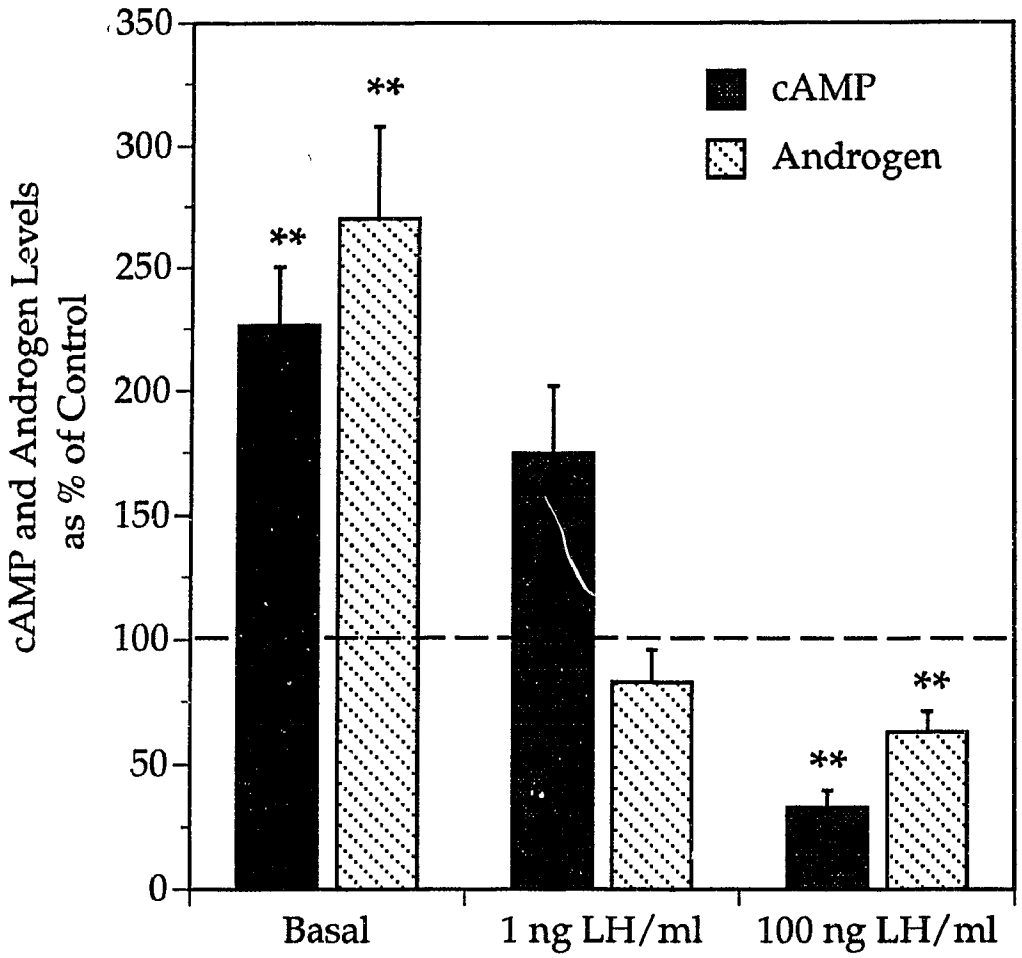

Figure 15.

Effect of 72 h of culture in the presence of IL-1 on basal and LH-stimulated cAMP and androgen release expressed as '% of Control'. Cells were cultured for 3 days with or without 100 U IL-1/ml and then further cultured for 4 h in the presence of either 1 ng LH/ml or 100 ng LH/ml. The culture medium was then collected in sample pots containing a phosphodiesterase inhibitor and immediatly frozen. Results are presented as '% of Control levels'. Non-standardized levels are given in the following three figures (Figure 16, 17, 18). *, $p<0.05$ and **, $p<0.01$ by two-tailed, one-sample t-test; n=6 for basal and 100 ng LH/ml and n=3 for 1 ng LH/ml.

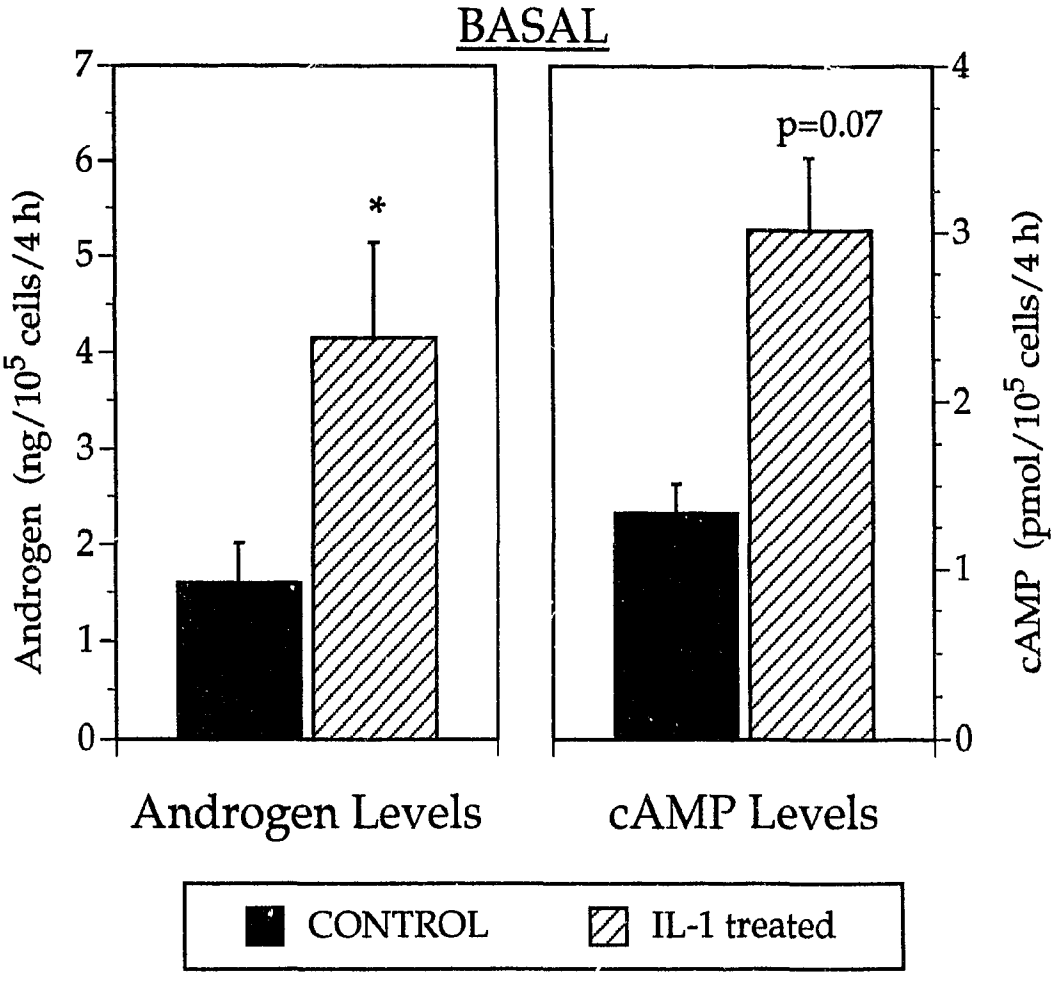

Figure 16.

Effect of IL-1 on Basal cAMP and androgen levels. Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml as described before. At the end of the culture period the medium was replaced and the cells were cultured for an additional 4 h period. The culture medium was collected in sample pots containing a phosphodiesterase inhibitor and immediatly frozen. *, $p<0.05$ by two tailed t-test; n=6.

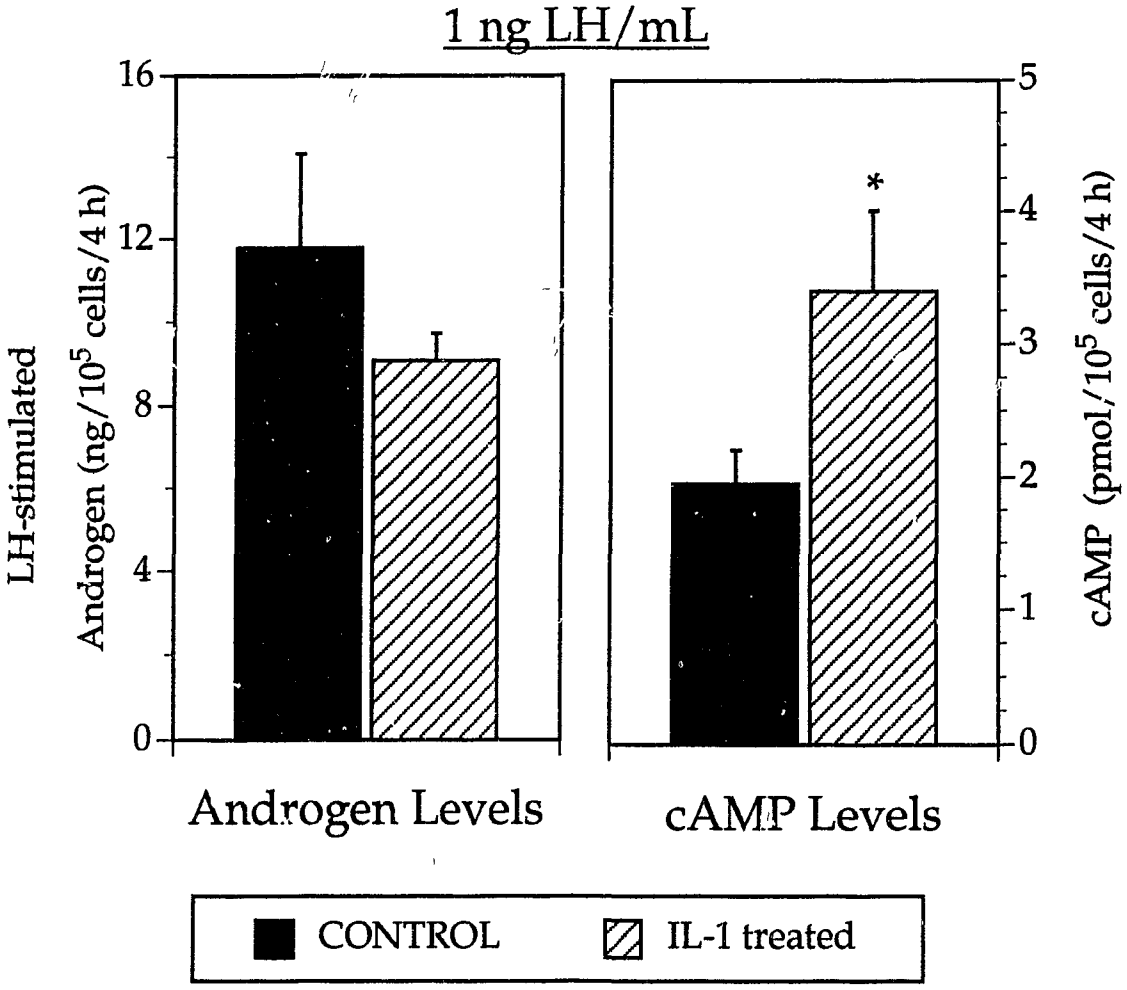

Figure 17.

Effect of IL-1 on cAMP and androgen levels in Leydig cell cultures stimulated with 1 ng LH/ml. Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml as described before. At the end of the culture period the medium was replaced and the cells were cultured for an additional 4 h period in the presence or absence of 1 ng LH/ml. The culture medium was then collected in sample pots containing a phosphodiesterase inhibitor and immediatly frozen. *, $p<0.05$ by two-tailed t-test; n=3.

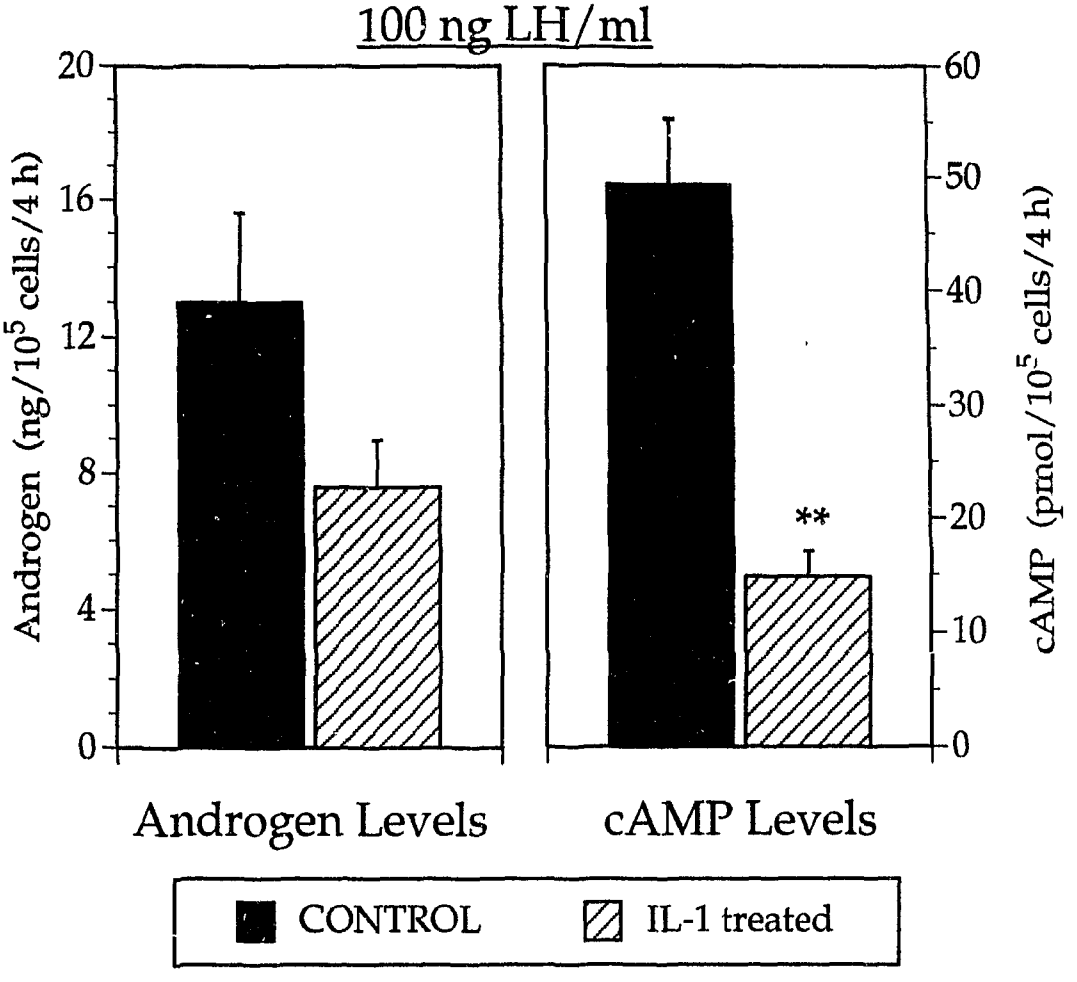

Figure 18.

Effect of IL-1 on cAMP and androgen levels in Leydig cell cultures stimulated with 100 ng LH/ml. Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml as described before. At the end of the culture period the medium was replaced and the cells were cultured for an additional 4 h period in the presence of 100 ng LH/ml. The culture medium was then collected in sample pots containing a phosphodiesterase inhibitor and immediatly frozen. **, $p<0.01$ by two-tailed t-test; n=6.

Effect of Pertussis Toxin on 4 h Basal Androgen and cAMP Levels in IL-1α-treated Leydig Cell Cultures

Figure 19 illustrates the effect of PTX (0.1 μg/ml) on basal androgen and cAMP levels in cells treated with IL-1α for 3 days expressed as % of Control and Figure 22 gives the untransformed mean response. Two-way analysis of variance on individual experiments revealed that there was a significant main effect of IL-1α on both basal androgen and cAMP levels ($p<0.01$) but the main effect of PTX was significant only for basal cAMP levels ($p<0.01$). There was a significant interaction between IL-1α and PTX treatments in all experiments ($p<0.05$). One-sample t-test on the combined data (expressed as % of Control) showed that PTX alone had no significant effect on basal cAMP and androgen levels. IL-1α alone had a significant stimulatory effect on basal cAMP and androgen levels ($p<0.01$ and $p<0.05$, respectively). The stimulatory effect of IL-1α on basal cAMP levels was further enhanced by PTX ($p<0.05$) but basal androgen levels were not further increased when PTX was combined with IL-1α. The statistical analysis of combined results reflected the responses in individual experiments.

Effect of Pertussis Toxin on 4 h LH-stimulated Androgen and cAMP Levels in IL-1α-treated Leydig Cell Cultures

In the presence of a submaximally effective concentration of LH (1 ng/ml) (Figure 20 as "% of Control" and Figure 22 for untransformed mean

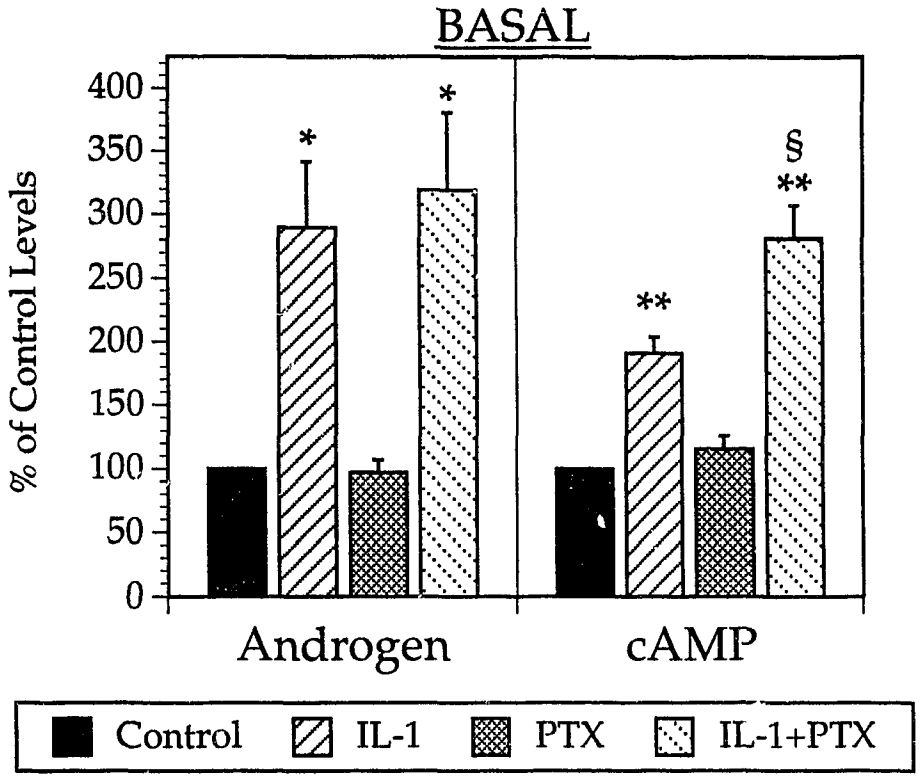

Figure 19.

Effect of PTX on IL-1-induced increase in basal androgen and cAMP release (as '% of Control'). Cells were cultured for 3 days in the presence or absence of IL-1 (100 U/ml) as previously described. Four hours (4 h) prior to the end of the culture period, PTX (0.1 μg/ml) was added to the culture medium. At the end of the culture period, the medium was replaced, PTX and IL-1 re-added where appropriate and the cells further cultured for a 4 h period. Samples were collected in pots containing a phosphodiesterase inhibitor. Mean androgen and cAMP concentrations in the culture medium are shown in Figure 22. *, $p<0.05$; **, $p<0.01$ compared to control (100%) by two-tailed, one-sample t-test and §, $p<0.05$ compared to IL-1 alone by two-tailed t-test, n=4.

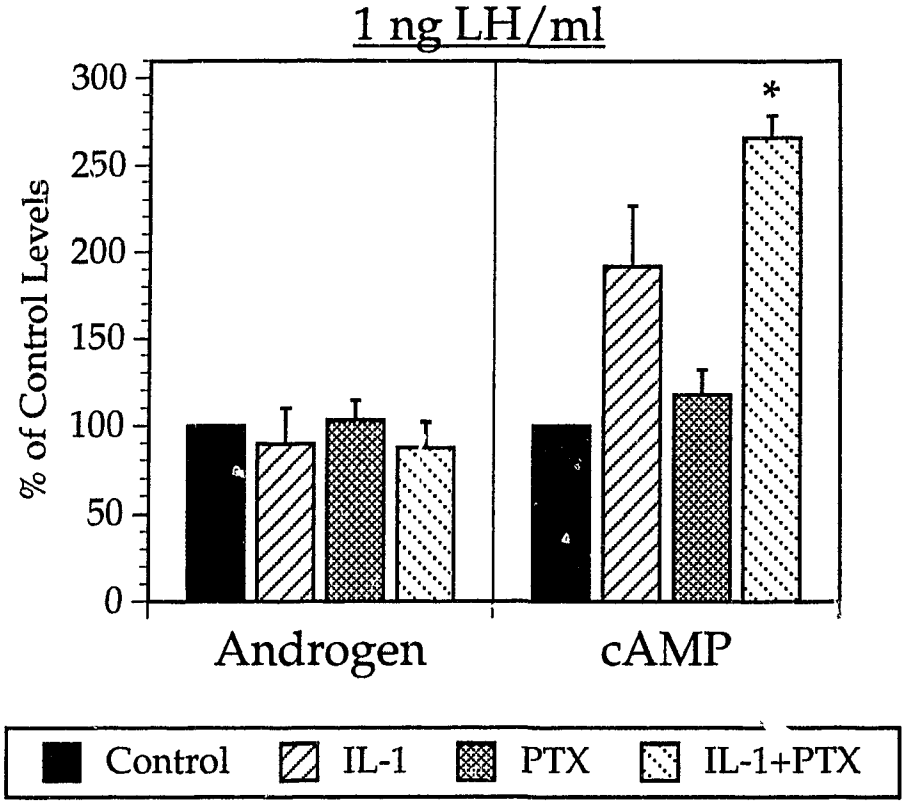

Figure 20.

Effect of PTX on IL-1-induced changes in androgen and cAMP release in cells stimulated with 1 ng LH/ml (as '% of Control'). Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml as previously described. Four hours prior to the end of the culture period 0.1 μg PTX/ml was added to the culture medium. At the end of the culture period, the medium was replaced, PTX and IL-1 re-added where appropriate and the cells further cultured for a 4 h period in the presence or absence of 1 ng LH/ml. Samples were collected in pots containing a phosphodiesterase inhibitor. Mean androgen and cAMP concentrations in the culture medium are shown in Figure 22.
*, $p<0.05$; compared to control (100%) by two-tailed, one-sample t-test, n=2.

response), the response to IL-1α and PTX was somewhat variable. In one of two identical experiments, IL-1α had an inhibitory effect on androgen release ($p<0.01$ by two-sided Dunnett t-test), accompanied by a stimulatory effect on cAMP levels ($p<0.01$ by two-sided Dunnett t-test). In that same experiment, PTX alone had a small stimulatory effect on both cAMP and androgen levels (($p<0.05$ by two-sided Dunnett t-test). The combination of PTX and IL-1α resulted in an enhancement of the effect of IL-1α alone, that is, the inhibitory effect on androgen levels and the stimulatory effect on cAMP levels were significantly greater with IL-1α + PTX than with IL-1 alone ($p<0.01$ by two-tailed t-test). In the other experiment, IL-1α had a significant stimulatory effect on cAMP levels ($p<0.01$ by two-sided Dunnett t-test) but no effect on androgen levels. PTX alone had no effect at all and the combination of PTX with IL-1α had the same effect as IL-1α alone.

In the presence of 100 ng LH/ml (Figure 21 for "% of Control" and Figure 22 for untransformed mean response), IL-α had an inhibitory effect on androgen release ($p=0.072$ on combined data by one-sample t-test but $p<0.01$ in 3 out of 4 experiments) as well as an inhibitory effect on cAMP levels ($p<0.01$ on combined data by one-sample t-test and $p<0.01$ in all individual experiments by Dunnett t-test). PTX alone had a stimulatory effect on cAMP levels ($p=0.09$ on combined data, $p<0.05$ in 3 out of 4 experiments) but no effect on androgen release. When PTX was added along with IL-1α it failed to

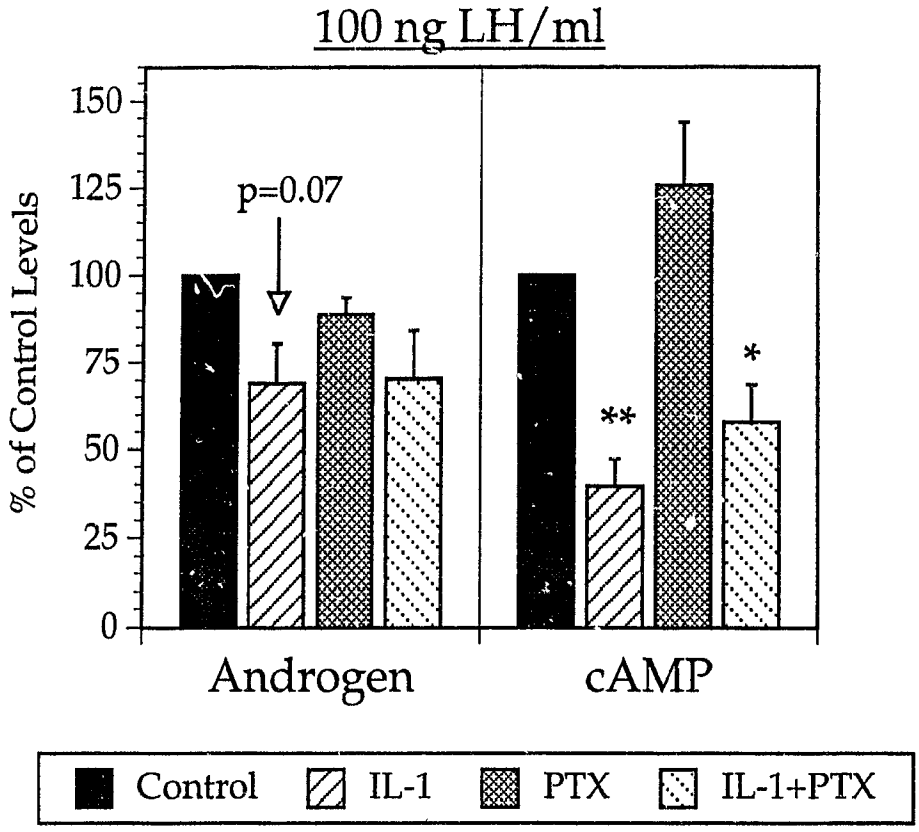

Figure 21.

Effect of PTX on the IL-1-induced inhibition of maximally LH-stimulated androgen and cAMP release (as '% of Control'). Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml as previously described. Four hours prior to the end of the 3-day culture period 0.1 µg PTX/ml was added to the culture medium. At the end of the culture period, the medium was replaced, PTX and IL-1 re-added where appropriate and the cells further cultured for a 4 h period in the presence of 100 ng LH/ml. Samples were collected in pots containing a phosphodiesterase inhibitor. Actual androgen and cAMP mean concentrations in the culture medium are shown in Figure 21. *, $p<0.05$; ** $p<0.01$ compared to control (100%) by two-tailed, one-sample t-test, n=4.

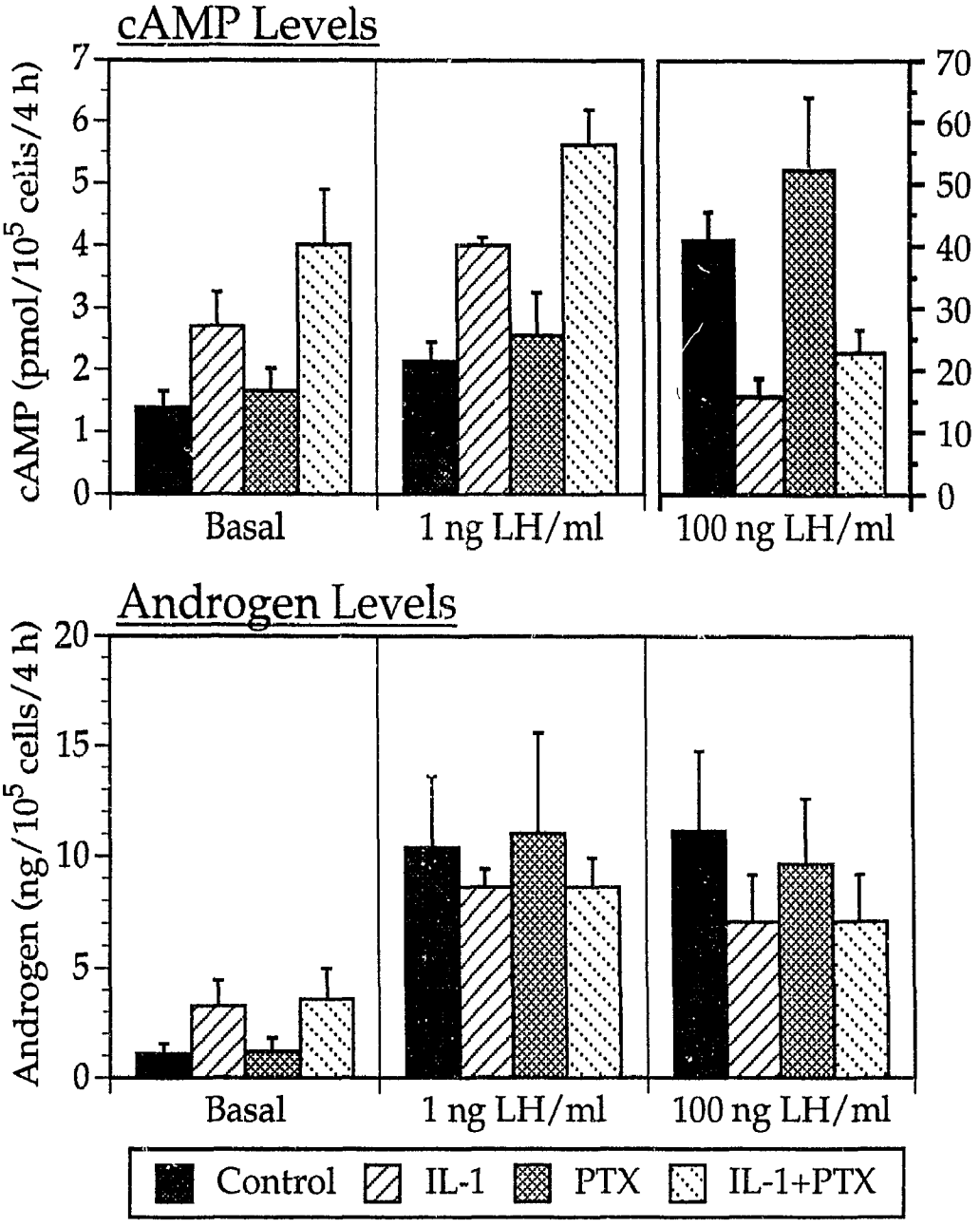

Figure 22.

Effect of PTX on androgen and cAMP release by IL-1-treated Leydig cells. This figure complements Figures 19, 20 and 21 by showing the actual levels of androgen and cAMP. Statistical analysis was done on the data expressed as '% of Control' and is summarized in Figures 19-21 as well as on individual experiments as discussed in the Results section.

block the inhibitory effect of IL-1α on both androgen and cAMP levels. It should be mentioned that in 2 out of 4 experiments cAMP levels were greater in PTX + IL-1α-treated cells than in IL-1α-treated cells ($p<0.01$ by Dunnett t-test) but that these changes in cAMP levels were not accompanied by a greater release of androgen.

The results from the experiments with PTX and IL-1α in the presence of submaximum and maximum concentration of LH indicate two things. First, PTX cannot block the inhibitory effect of IL-1α on LH-induced cAMP levels. Second, the changes in cAMP levels are not always followed by parallel changes in androgen levels; in the presence of 1 ng LH/ml the IL-1α-induced increase in cAMP levels was accompanied by either no change or by a decrease in androgen release and in the presence of 100 ng LH/ml, PTX, when combined with IL-1α, could increase cAMP levels but these changes were not reflected by changes in androgen release.

Effect of IL-1α and the Cyclooxygenase Inhibitors Indomethacin and ASA on Basal and LH-stimulated Androgen Release

Many of the documented effects of IL-1 have been shown to involve the production of arachidonic acid metabolites and, in particular, prostaglandins (Dinarello, 1986). Experiments were therefore initiated to examine the possibility that the cyclooxygenase pathway of arachidonic acid metabolism

may be involved in the action of IL-1α on Leydig cell steroidogenesis.

Indomethacin at a concentration of 100 μM was first tested as this concentration had been previously reported to be effective in Leydig cell incubations (Didolkar & Sundaram, 1989; Lin, 1985; Sullivan, Abayasekara & Cooke, 1988). Leydig cells were prepared as previously described and cultured in the continuous presence or absence of 100 μM indomethacin and/or 100 U IL-1α/ml. The effect of those treatments on 24 h basal androgen release are shown in Figure 23 (top panel for data expressed as % of Control and bottom panel for untransformed data). Indomethacin had a stimulatory effect on basal androgen release ($p<0.05$ day 1, $p<0.05$ day 2, $p=0.06$ day 3 by two-tailed, one-sample t-test) and the effect was relatively constant over the 3 day period at 2 to 2.5 fold increase. IL-1α had the expected stimulatory effect on basal androgen release but indomethacin failed to reduce this effect. In fact, there was a slight but significant increase of the stimulatory effect of IL-1α on day 1 and day 2 ($p<0.05$ by two-tailed t-test) when indomethacin was also present.

As shown on Figure 24 (and Figure 25 for untransformed data), 4 h basal androgen release at the end of the 72 h culture period was enhanced by IL-1α and IL-1α + Indomethacin ($p<0.01$). In addition, and in contrast to the results for 24 h basal, indomethacin partially inhibited the stimulatory effect of IL-1α on 4 h basal androgen release ($p<0.01$).

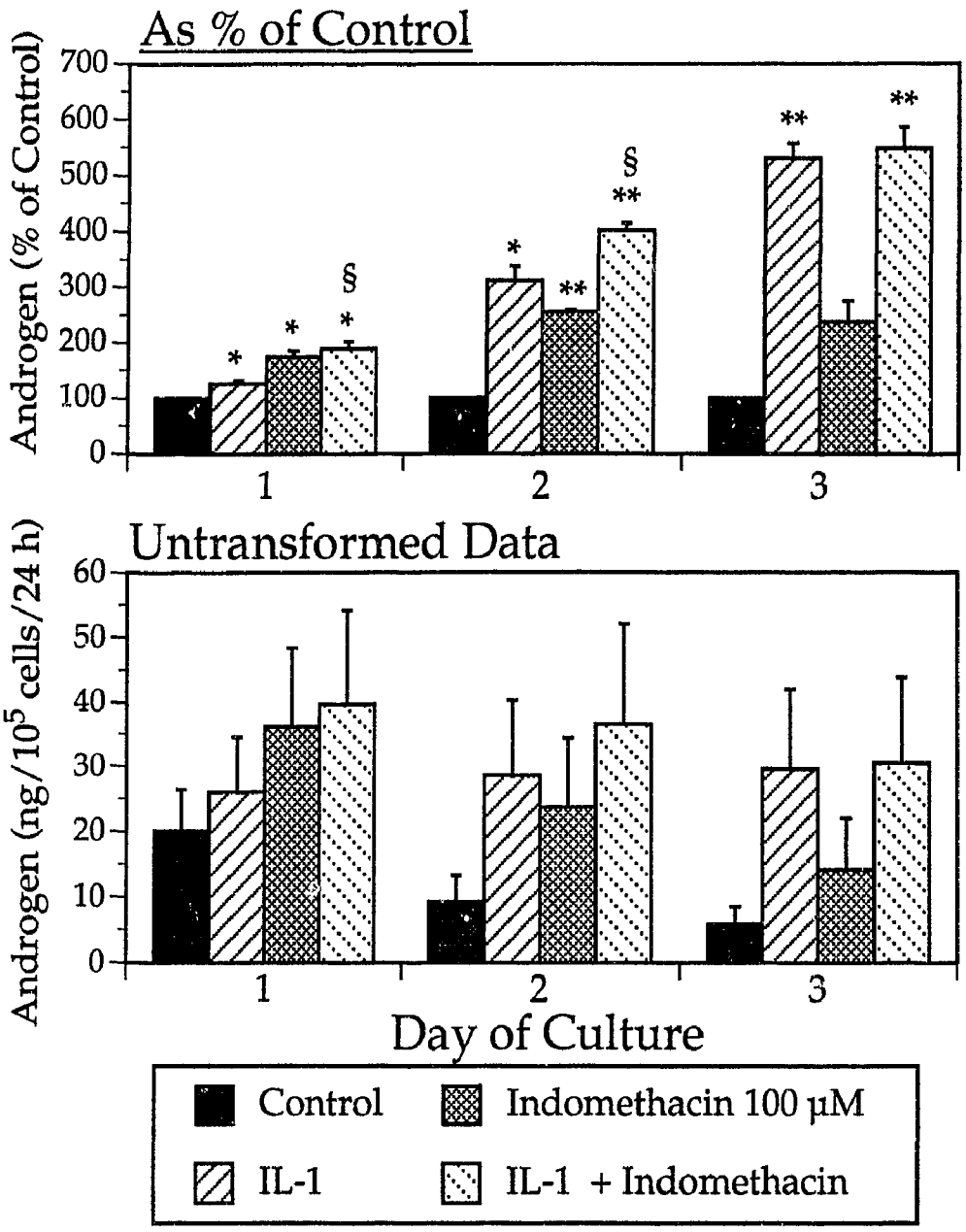

Figure 23.

Effect of 100 µM Indomethacin, a cyclooxygenase inhibitor, on the stimulatory effect of IL-1 on basal androgen release. Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml and/or 100 µM Indomethacin. The medium was changed every 24 h and fresh Indomethacin and IL-1 added where appropriate. The top panel represent the response to the various treatments as a percentage of the Control response on each day. The bottom panel shows the non-normalized data. *, and §, $p<0.05$; **, and §§, $p<0.01$ compared to Control (*) or compared to IL-1 alone (§), n=4.

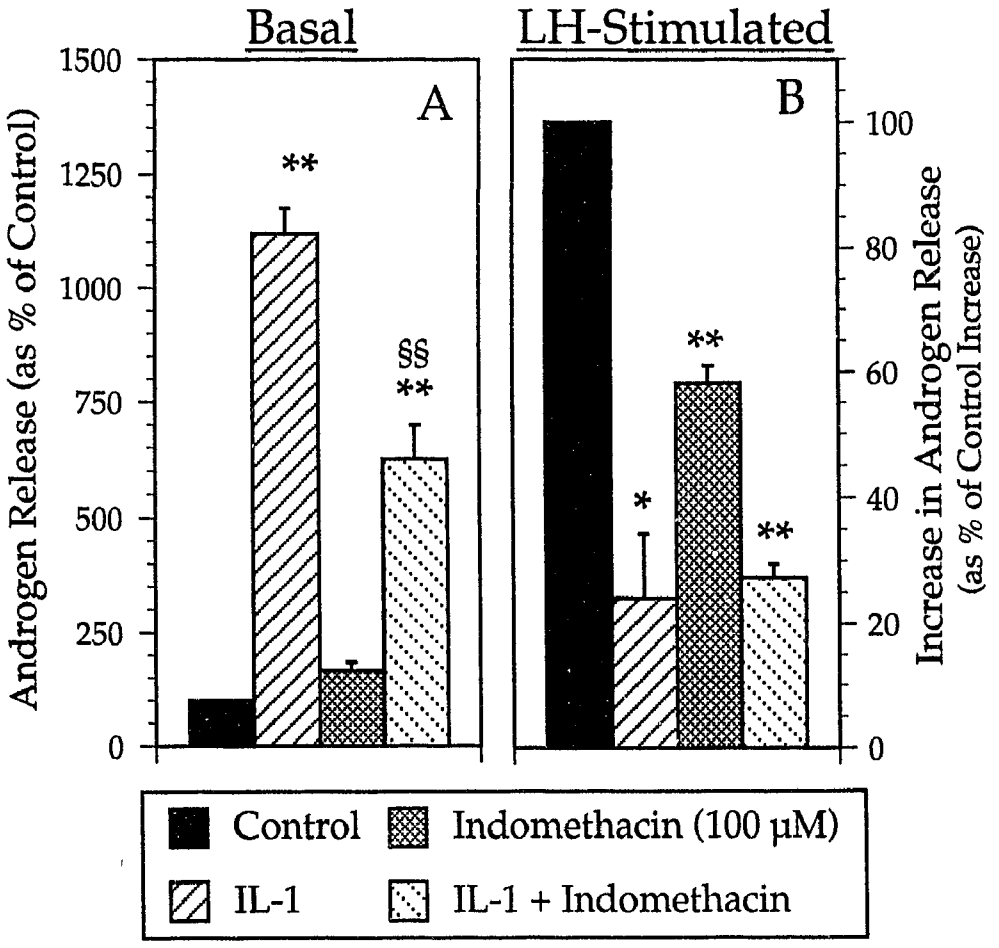

Figure 24.

Effect of 100 µM Indomethacin on IL-1-induced changes in basal and LH-stimulated androgen release (as '% of Control'). Cells were cultured for 3 days in the presence or absence of IL-1 (100 U/ml) and/or Indomethacin (100 µM) as described previously. At the end of the 3 days the cells were further cultured for 4 h in the presence or absence of LH (100 ng/ml) and appropriate treatment. Values are the mean ± SEM of 3 experiments. Basal androgen release is expressed as '% of Control' and the response to LH is presented as the increase in androgen release over basal as % of increase in Control group. *, $p<0.05$; **, §§, $p<0.01$ compared to Control (*) by two-tailed, one-sample t-test, or compared to IL-1 alone (§) by t-test. Mean, untransformed data for these experiments can be seen in Figure 25.

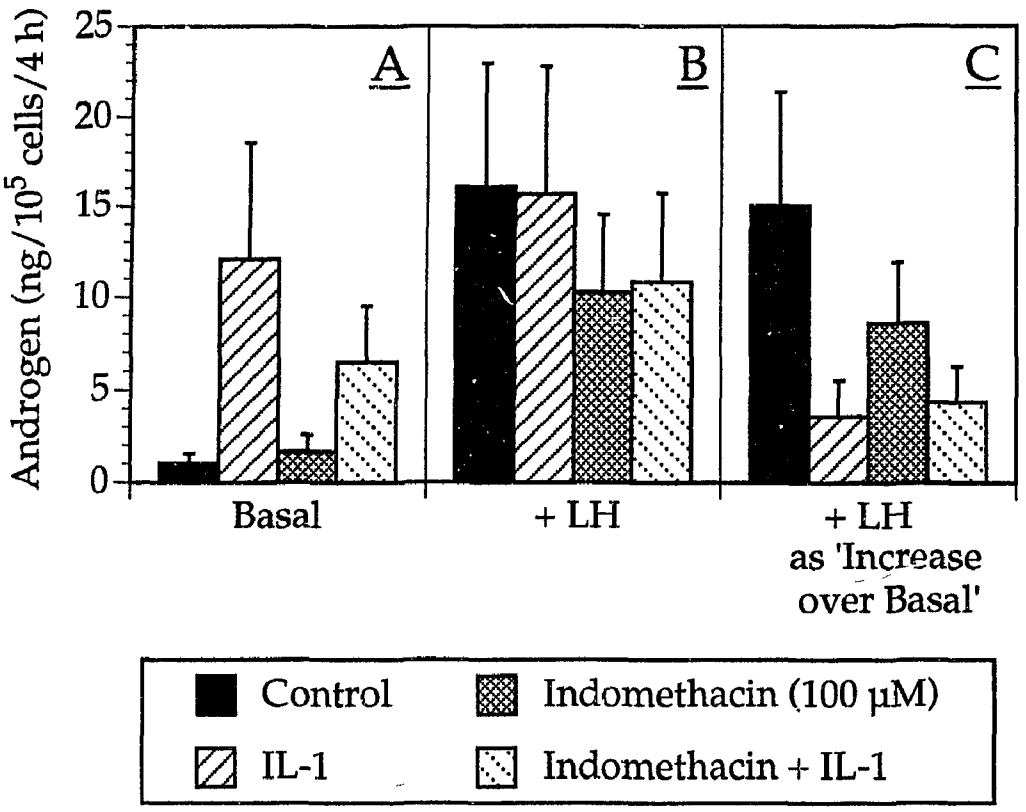

Figure 25.

Four hour basal and LH-stimulated androgen release by cells treated with IL-1 and/or 100 µM Indomethacin for 3 days. This figure complements Figure 23 which shows the results from the same experiments in a normalized form. The untransformed data for 4h Basal (A) and LH-stimulated androgen release (B) is seen here, as well as the increase in androgen release over basal levels (C) induced by LH (100 ng/ml) in the various treatment group. Statistical analysis was performed on the standardized data and is summarized in Figure 23 (n=3).

The increase in androgen release in response to 100 ng LH/ml was inhibited by IL-1α ($p<0.01$) and, to a lesser extent, by indomethacin ($p<0.05$). The combination of indomethacin with IL-1α did not further inhibit the increase in androgen release over basal compared to IL-1α alone nor did it reverse it. The actual concentration of androgen in the culture medium (as opposed to the 'increase over Basal') was lowest with indomethacin alone or in combination with IL-1α (see Figure 25).

The concentration of indomethacin used (100 μM) in these experiments was later found to be high enough to also potentially inhibit the lipoxygenase pathway of arachidonic acid metabolism (Rainsford, 1988). When it was used at a lower concentration (20 μM), indomethacin failed to induce any changes in 24 h basal androgen release or to interact with IL-1α (Table 7). There was also no effect on 4 h basal androgen production. The same absence of effect was observed when ASA (400 μM), another cyclooxygenase inhibitor, was used. Furthermore, both inhibitors failed to induce any changes in 4 h LH-stimulated androgen release or to modify the effect of IL-1α. At these concentrations, these inhibitors of the cyclooxygenase pathway of arachidonic acid metabolism are believed to be quite specific (Rainsford, 1988).

400 μM ASA

	IL-1	ASA (400μM)	ASA + IL-1
DAY 1	130.4 ± 10.3	119.9 ± 6.7	128.7 ± 17.3
DAY 2	327.2 ± 45.6 *	103.6 ± 11.6	300.0 ± 36.3
DAY 3	508.7 ± 84.2 *	102.1 ± 10.6	545.1 ± 82.0
4h BASAL	1292.4 ± 104.6 **	144.6 ± 35.9	1461.1 ± 152.0
4h LH-STIMULATED	30.1 ± 17.9 (p=.06)	111.4 ± 7.7	32.8 ± 10.1

20 μM Indomethacin

	IL-1	INDOMETHACIN (20μM)	INDOMETHACIN + IL-1
DAY 1	125.6 ± 8.6	107.9 ± 8.0	128.7 ±12.4
DAY 2	336.9 ± 33.7 **	121.9 ± 15.3	410.1 ± 44.6
DAY 3	467.2 ± 72.6 *	122.4 ± 11.0	591.0 ± 120.9
4h BASAL	1194.7 ± 122.6 **	147.4 ± 7.4	1299.5 ± 162.7
4h LH-STIMULATED	30.7 ± 13.1 *	121.5 ± 6.4	33.9 ± 10.7

Table 7.

Effects of 400 μM ASA and 20 μM Indomethacin on the IL-1-induced changes in basal and LH-stimulated androgen release. Cells were cultured for 3 days in the presence or absence of IL-1 (100 U/ml) and/or ASA or Indomethacin as previously described. Values represent the means of 3 or 4 separate experiments. The data are expressed as percent of Control response or, in the case of LH-stimulated androgen release, as the increase over basal as % of Control increase. *, $p<0.05$; **, $p<0.01$ compared to Control (100%). There were no differences between the groups treated with IL-1 alone and those treated with a IL-1 + ASA or IL-1 + Indomethacin.

Effect of the Lipoxygenase Inhibitor NDGA and IL-1α on Basal and LH-stimulated Androgen Release

The difference in the results obtained with 100 μM and 20 μM indomethacin suggested that the lipoxygenase pathway of arachidonic acid metabolism could be involved in IL-1α action on Leydig cells. To test this hypothesis nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor was used. Cells were cultured in the continuous presence or absence of IL-1α (100 U/ml) and/or NDGA (10 μM) for three days, followed by 4 h in the presence or absence of these treatments and 100 ng LH/ml. Changes in 24 h basal androgen release are shown in Figure 26. NDGA alone had a significant stimulatory effect on 24 h basal androgen release on day 1 ($p<0.01$) and day 2 ($p<0.05$). On day 3, NDGA had no effect on androgen release. On day 1, cells cultured in the presence of IL-1α + NDGA released the same increased amount of androgen as those treated with NDGA alone. However, on day 2 and day 3 NDGA partially blocked the stimulatory effect of IL-1α on basal androgen release (interaction between effects of IL-1α and NDGA; $p< 0.01$ and 0.05 for days 2 and 3, respectively). The gradual disappearance of the stimulatory effect of NDGA and the decrease in the effect of IL-1α in the presence of NDGA could not be attributed to cell loss; IL-1α, NDGA or their combination did not alter the DNA content of the cultures (Table 4).

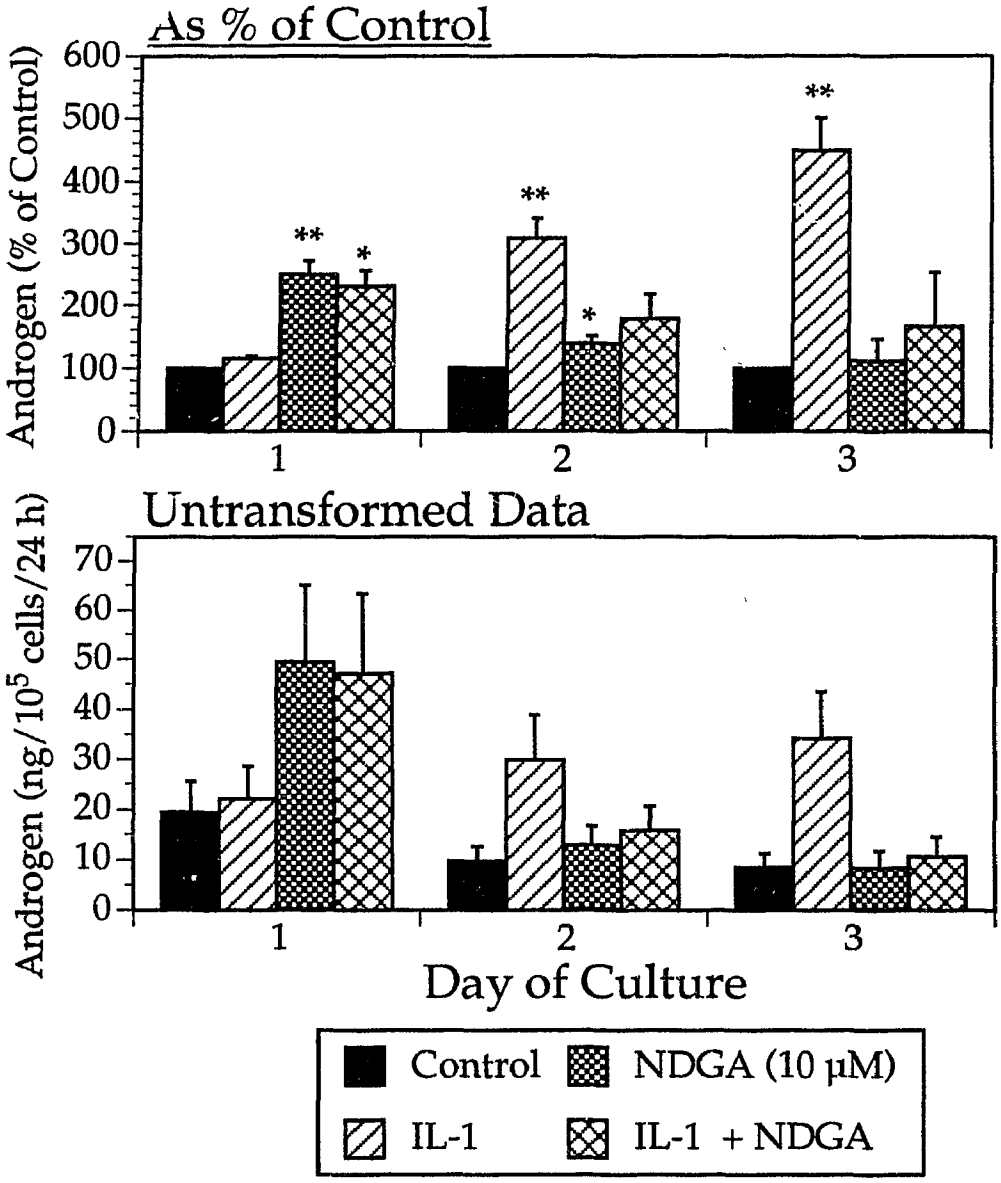

Figure 26.

Effect of NDGA, a lipoxygenase inhibitor, on the stimulatory effect of IL-1 on basal androgen release. Cells were cultured for 3 days in the presence or absence of IL-1 (100 U/ml) and/or NDGA (10 µM). The medium was changed every 24 h and fresh NDGA and IL-1 added where appropriate. The top panel represent the response to the various treatments as a percentage of the Control response on each day. The bottom panel shows the non-normalized data. *, $p<0.05$; **, $p<0.01$ compared to Control (100%) by two-tailed, one-sample t-test, n=4.

The suppression by NDGA of the stimulatory effect of IL-1α on basal androgen release was still present during the 4 h test period on day 3 of culture (Figure 27). Both NDGA and IL-1α inhibited the LH-induced increase in androgen release over basal ($p<0.05$ and $p<0.01$, respectively). The combination of IL-1α and NDGA resulted in a similar inhibition of the 'increase over basal' as that seen with IL-1 alone. When looking at the actual concentration of androgen rather than the 'increase over basal' (Figure 28) it can be seen that NDGA treated cells released significantly less androgen than IL-1α treated cells and that the combination of NDGA with IL-1α resulted in the same low levels of androgen as with NDGA alone.

Changes in Progesterone Release by Leydig Cells in Culture in Response to IL-1α and NDGA

Results from the experiments using 25-OH cholesterol and pregnenolone described above as well as results published by others (Fauser et al., 1989; Verhoeven et al., 1988) indicate that IL-1α exerts at least part of its inhibitory effect on LH-stimulated androgen release at the level of the C17-20 lyase/17α-hydroxylase enzyme. An inhibition of the metabolism of C21 steroids to C19 steroid would be expected to be accompanied by an accumulation of C21 steroids. To verify this hypothesis we measured the release of progesterone (C21) by IL-1α-treated Leydig cell in the presence or absence of LH. Samples from two experiments using NDGA were also assayed.

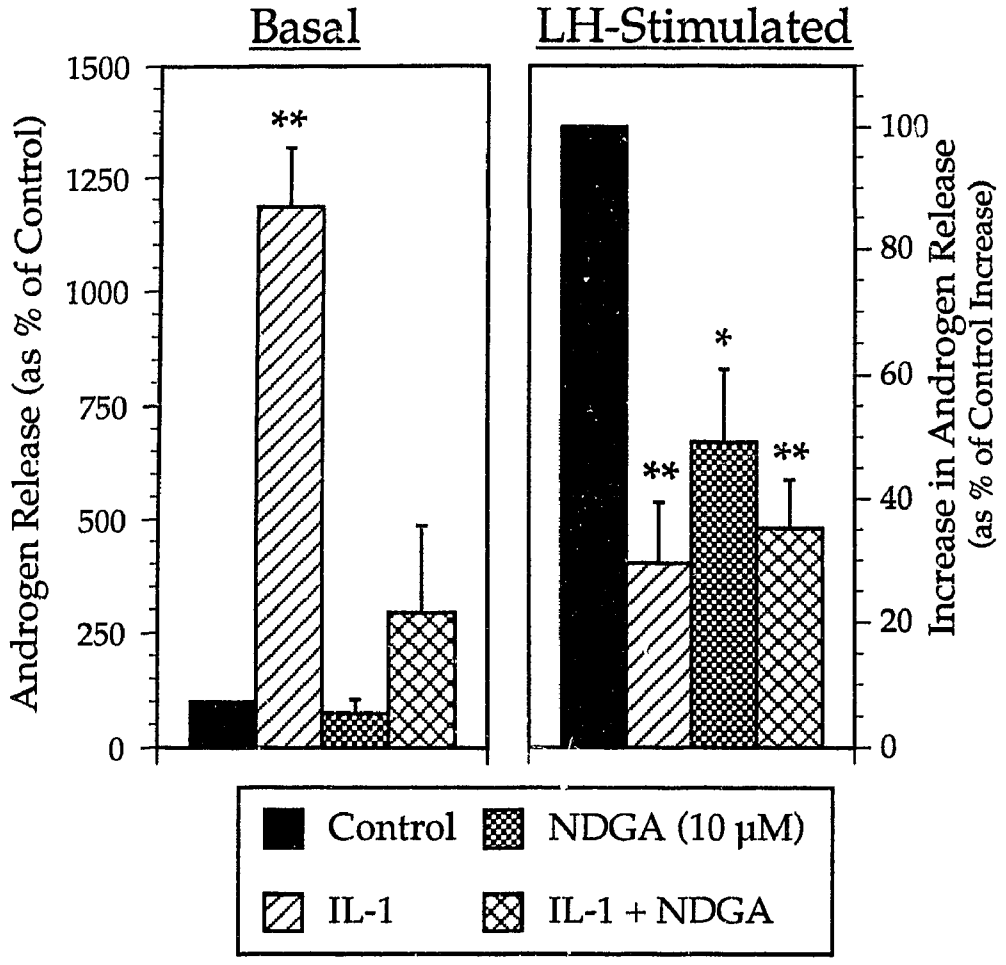

Figure 27.

Effect of NDGA on IL-1-induced changes in Basal and LH-stimulated androgen release. Cells were cultured for 3 days in the presence or absence of IL-1 (100 U/ml) and/or NDGA (10 µM) as described previously. At the end of the 3 days, the cells further cultured for 4 h in the presence or absence of LH (100 ng/ml) and appropriate treatment. Values are the mean ± SEM of 4 separate experiments. Basal androgen release is expressed as '% of Control' and the androgen response in the presence of LH is presented as the increase in androgen release over Basal as % of increase in the control group. *, $p<0.05$; ** $p<0.01$ compared to Control (100%) by two-tailed, one-sample t-test. Mean, untransformed data for these experiments can be seen in Figure 28.

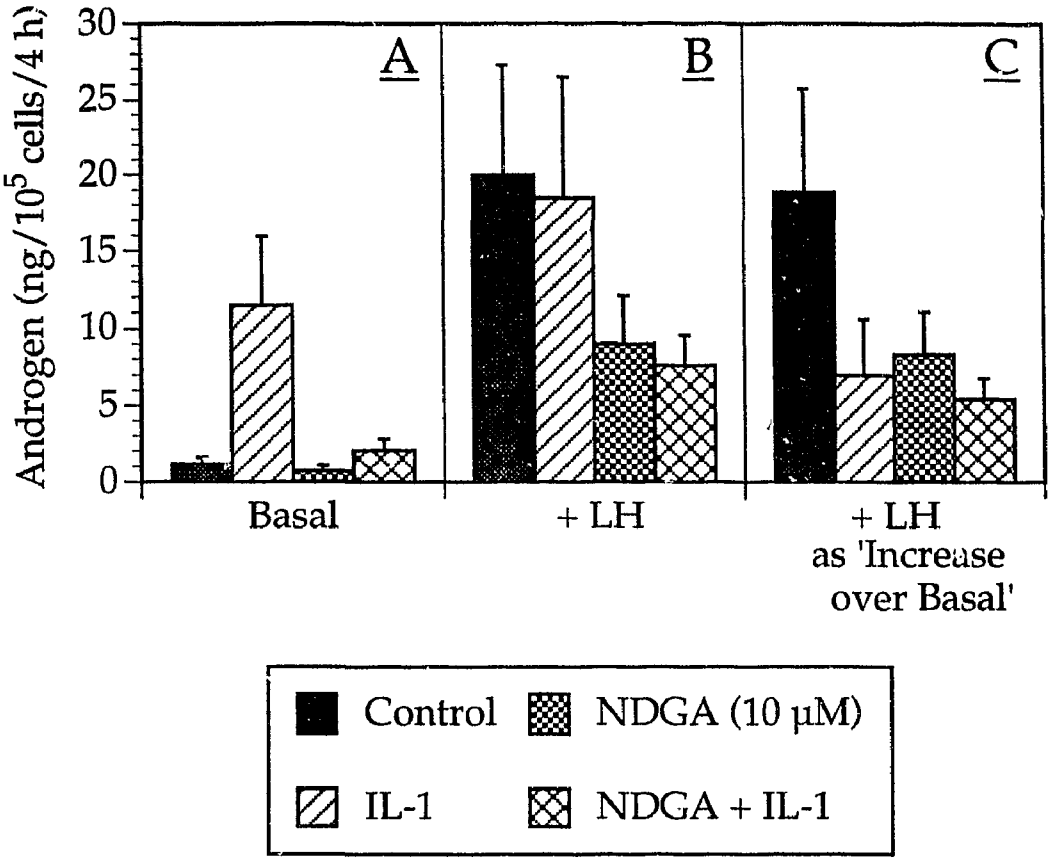

Figure 28.

Four hour Basal and LH-stimulated androgen release by cells treated with IL-1 and/or 10 μM NDGA for 3 days. This figure complements Figure 27 which shows the results from the same experiments in a normalized form. The untransformed data for 4 h basal (A) and LH-stimulated androgen release (B) is seen here, as well as the increase in androgen release over basal levels (C) induced by LH (100 ng/ml) in the various treatment group. Statistical analysis was performed on the standardized data and is summarized in Figure 27 (n=4).

Mean 4 h basal and LH-stimulated progesterone release in control and IL-1α treated cells are seen in Figure 29. This figure also includes mean androgen levels for the same experiments (inset). Because of the large variability in levels between experiments the results of statistical analysis will be described on a per experiment basis rather than on combined data. Under control conditions, progesterone levels were very low and often near or below the limit of our assay. When levels were below the limit of the assay basal progesterone levels were assigned the value of the last standard and the progesterone release by IL-1α-treated cells was compared to that value using a one-sample t-test. In nearly all the experiments where basal progesterone levels were measured, IL-1α-treated cells released significantly ($p<0.05$ for 5 out of 6 experiments, two-tailed t-test) more progesterone than control cells although the magnitude of the increase was quite variable (range = 1.4 fold to an 87 fold increase). IL-1α induced both an increase in basal androgen levels (see inset Figure 29) and in progesterone levels.

Stimulation of androgen release by LH was also accompanied by an increase in progesterone release in all experiments ($p<0.01$). In 3 out of 5 experiments there was a further increase in progesterone release with IL-1α ($p<0.01$ and $p<0.05$). The increase ranged from 1.7 to 3.4 times control LH-stimulated levels. However, in 2 experiments, despite significant and marked inhibition of androgen release, progesterone levels did not change significantly.

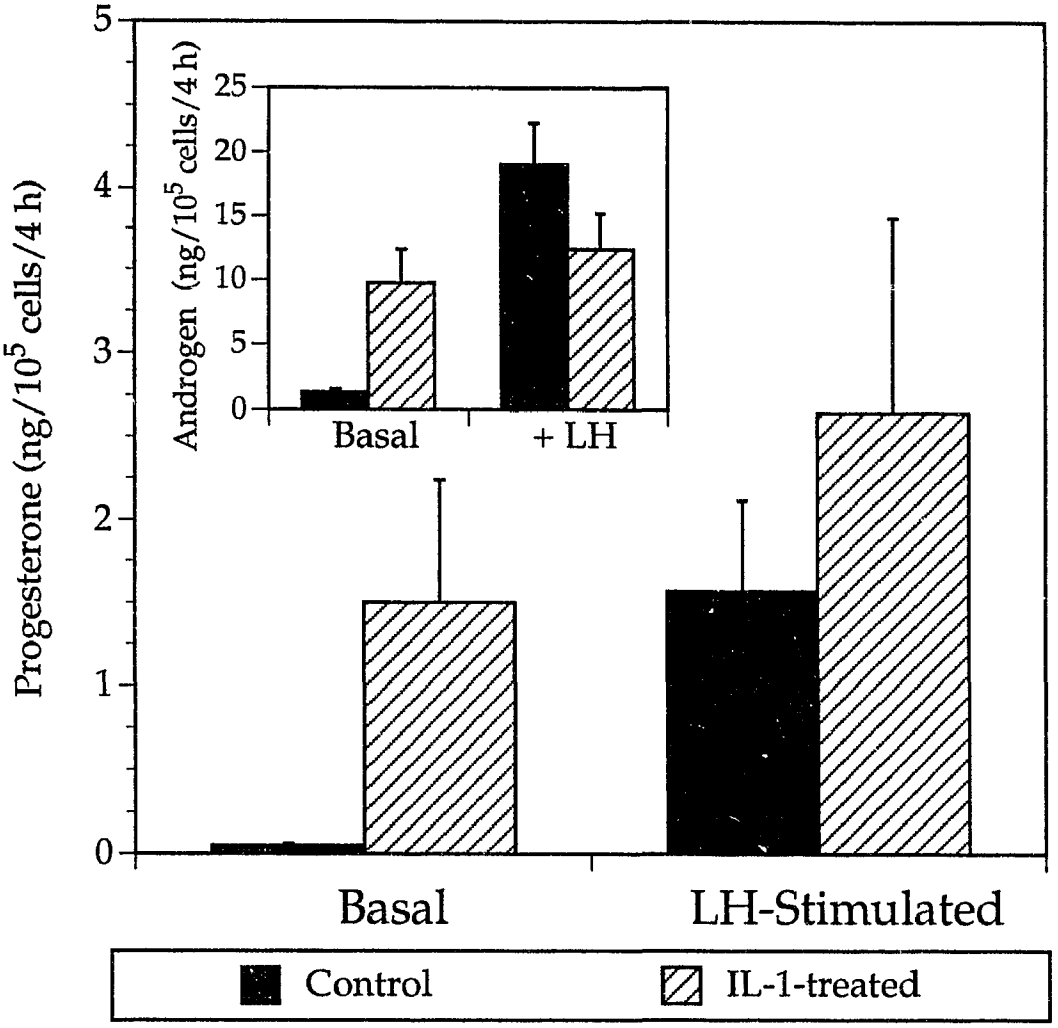

Figure 29.

Effect of IL-1 on Progesterone release by cultured Leydig cells. Cells were cultured for 3 days in the presence or absence of IL-1 (100 U/ml) as previously described and then incubated for an additional 4 h in the presence or absence of LH (100 ng/ml). Values represent progesterone released in the culture medium during that period and are the means ± SEM of 5-6 separate experiments. The inset shows the mean ± SEM androgen release in the same 5-6 experiments. Mean basal androgen release was significantly increased by IL-1 ($p<0.05$) and, mean LH-stimulated androgen release expressed as % of control 64.4% ± 6.4%) was significantly inhibited by IL-1 ($p<0.01$, two-tailed, one-sample t-test). Statistical analysis of progesterone release was done on individual experiments and results are described in the text.

Progesterone levels were measured in samples from two of the experiments using NDGA. Figure 30 shows the results from one experiment but both experiments gave similar results. In both cases, NDGA alone was without effect on 4 h basal and LH-stimulated progesterone levels but completely prevented the increase in progesterone induced by IL-1α in both basal and LH-stimulated conditions. Parallel changes in androgen release were seen, with NDGA having no effect on basal androgen release but blocking the stimulatory effect of IL-1α. The inhibitory effect of NDGA on LH-stimulated androgen release was not accompanied by an increase in progesterone levels. This suggests that the inhibitory effect of NDGA lies at step(s) prior to progesterone formation. Cells treated with NDGA + IL-1α release the same lower amounts of progesterone as those treated with NDGA alone. Androgen levels in the NDGA and NDGA + IL-1α treated groups were also reduced but were not different from each other, as was described in a previous section.

Effect of Culturing Leydig Cells in Testicular Macrophage Conditioned Medium (TMCM) on Basal Androgen Release.

Leydig cells were cultured for 3 days in either normal supplemented medium or testicular macrophage conditioned medium (TMCM). The TMCM was supplemented with insulin, vit E/DMSO and Pen-strep at the time it was used for the Leydig cell culture. As for all cultures, the medium was replaced every 24 h.

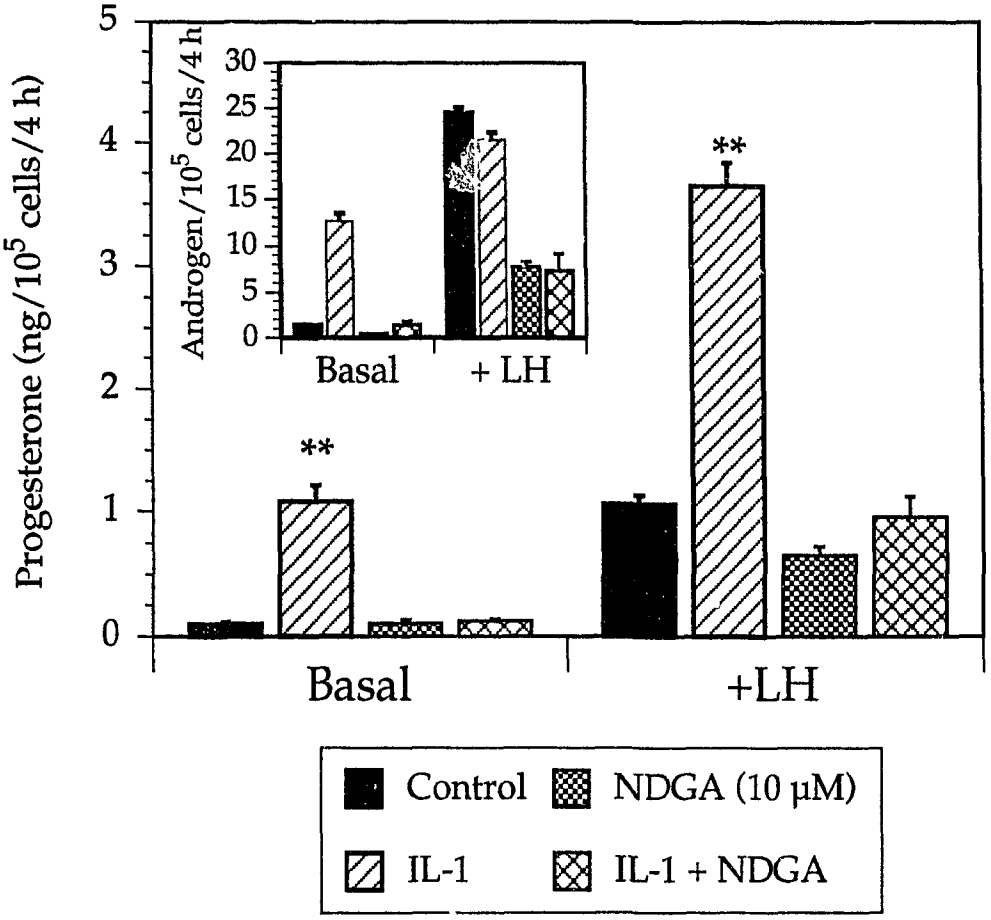

Figure 30.

Effect of IL-1 and NDGA or their combination on basal and LH-stimulated progesterone release. Cells were cultured for 3 days in the presence or absence of 100 U IL-1/ml and/or 10 μM NDGA and then cultured for an additional 4 h in the presence or absence of LH (100 ng/ml). Values are the means ± SEM of 4 replicate wells from one of the two NDGA experiments assayed for progesterone levels and are representative of results in that other experiment. The inset shows the levels of androgen released in the medium in that experiment. **, $p<0.01$ compared to control by two-tailed t-test.

The effect of TMCM on 24 h basal androgen release is presented in Figure 31. The response expressed as % of Control (panel A) and the actual androgen levels (panel B) are both shown. Statistical analysis was done on both sets of data. On day 1, there was no significant effect of TMCM but on day 2 and 3 cells treated with TMCM released significantly more androgen than cells cultured in control medium ($p<0.05$, $n=7$ and $n=3$ for day 2 and day 3, respectively). The untransformed data (panel B) show that androgen release declined over time in cells cultured in control medium but that it changed very little over the 3-day period in cells cultured in TMCM. The difference in androgen levels between the control and TMCM-treated groups reached significance on day 3.

Effect of Culturing Leydig cells in TMCM on LH-stimulated Androgen Release

At the end of the 3-day culture, the media were replaced, the cells treated with 100 ng LH/ml and cultured for an additional 4 h (Figure 32). Basal androgen release was still elevated in TMCM-treated cultures although the difference did not reach statistical significance ($p=0.066$ by two-tailed, paired t-test and $p=0.056$ by two-tailed, one-sample t-test, for data expressed as % of Control). TMCM-treated cells released 3.46 ± .61 times more androgen than Control cells under basal conditions. TMCM-treated cells released significantly more androgen in reponse to LH than control cells ($p<0.01$ by two-tailed

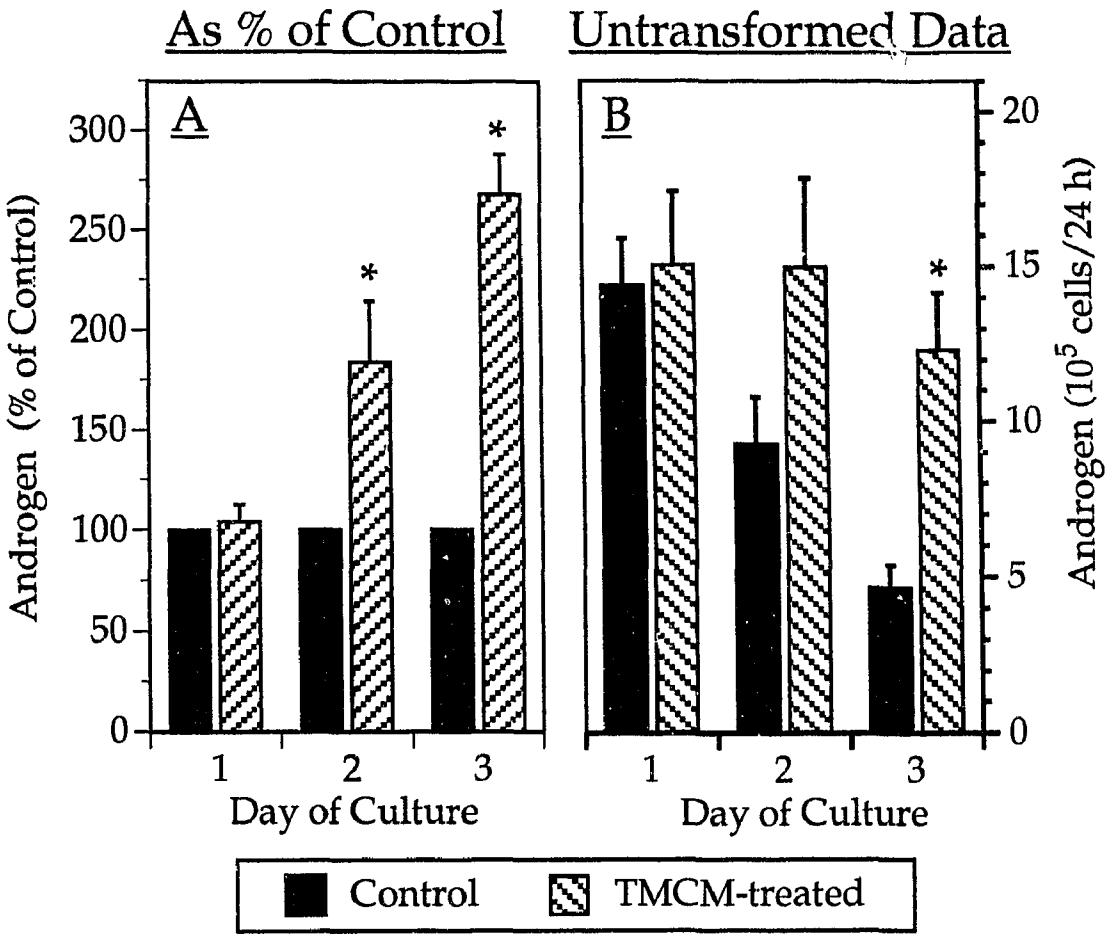

Figure 31.

Effect of testicular macrophage conditioned medium (TMCM) on basal androgen release by Leydig cells in culture. TMCM was prepared as described in the Materials and Methods and used as culture medium for Leydig cells culture. The medium was replaced every 24 h. Values are the mean ± SEM of 4, 7 and 3 separate experiments for day 1, 2, and 3, respectively. In Panel A, the response to TMCM is presented as '% of Control'. Panel B represents the actual amount of androgen released in the medium by Control and TMCM-treated cells. *, $p<0.05$ compared to Control by two-tailed, one-sample t-test for Panel A and by two-tailed t-test for Panel B.

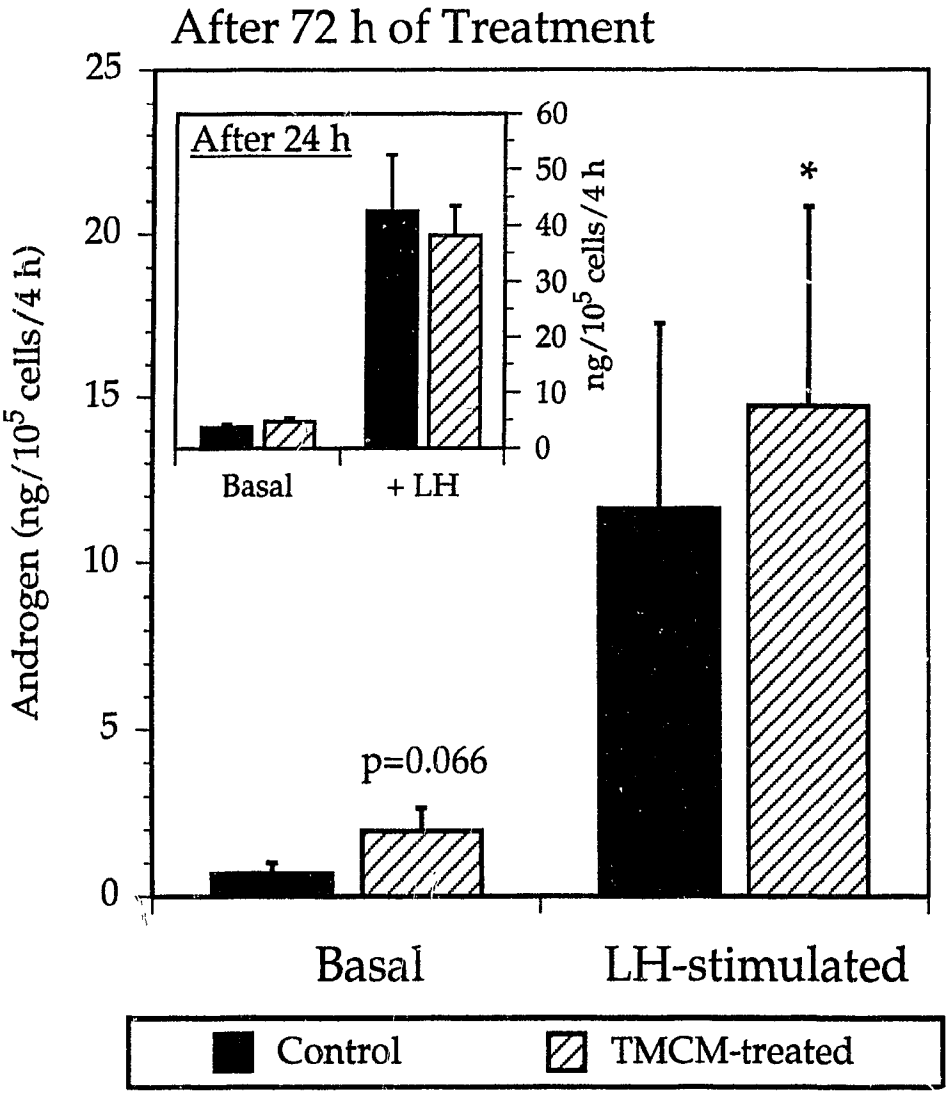

Figure 32.

Effect of testicular macrophage conditioned medium (TMCM) on basal and LH-stimulated androgen release by Leydig cells in culture. TMCM was prepared as described in the Materials and Methods and used as culture medium for Leydig cells culture. The medium was replaced every 24 h. At the end of the culture period the medium was replaced and the cells incubated for an additional 4 h in the presence or absence of LH (100 ng/ml). Values are the means ± SEM of 3 separate experiments. *, $p<0.05$ compared to Control by paired, two-tailed t-test. The inset illustrates the lack of changes in androgen release after 24h of culture in the presence or absence of TMCM. (n=4)

paired t-test on non-transformed data) but the increase was not as large as that seen for 4 h basal (1.46 ± .22 times Control levels).

When the 4 h LH-stimulation was performed after only 24 h of exposure to TMCM there was no difference in androgen release between the Control and TMCM-treated cells (inset of Figure 32). Similarly, 4 h Basal androgen release was unaffected.

Variability in the Effect of TMCM on Leydig Cell Steroidogenesis. Relationship to the Collagenase used for Cell Dispersion

While the first seven experiments with TMCM gave quite consistent results, the response to TMCM became erratic in the subsequent experiments. Table 8 lists all the experiments conducted on the effect of TMCM on 24 h basal, and 4 h basal and LH-stimulated androgen release. For each experiment the direction (up or down arrows) and the magnitude (expressed as % of Control) of the changes is given. Standard errors were omitted for clarity of presentation. The time at which these levels were measured (in 'day of culture with TMCM') is indicated. In addition, and most importantly, the batch of collagenase used to prepare the macrophages and the Leydig cells is specified for each experiment (a letter, rather than the full batch number was used for the table). Given the small number of experiments for each batch or combination of batches, firm conclusions cannot be drawn from this table.

As % of Control

EXP. #	1	2	3	4	5	6	7	8	9	10	11
ENZ. for MØ	a	a	a	a	a	a	b	b	c	c	e
ENZ. for L¢	a	a	a	a	a	a	b	c	d	d	d
24 h BASAL											
DAY 1	...	...	...	↑127	104	↓83	107	105	↓62	↓76	↓65
DAY 2	↑261	↑161	↓85	...	↑224	↑121	↑151	...	↓65	↓81	↓75
DAY 3	...	...	...	...	↑307	↑239	↑258	...	↓89	93	...
DAY 4	...	...	...	...	...	...	...	...	...	98	...
4 h BASAL											
DAY 1	↑120	↑162	96	↑127	...	...	...	↓47	...	...	...
DAY 2	...	...	...	...	...	...	...	...	...	...	↓52
DAY 3	...	...	...	...	↑248	↑333	↑457	...	↓47	...	...
DAY 4	...	...	...	...	...	...	...	...	...	↑307	...
4 h LH-STIM'D											
DAY 1	↑125	96	↓67	101	...	...	...	92	...	...	...
DAY 2	...	...	...	...	...	...	...	...	...	...	↓86
DAY 3	...	...	...	...	↑118	↑130	↑190	...	91	...	...
DAY 4	...	...	...	...	...	...	...	...	...	↓59	...

Table 8.

Effect of batch of collagenase on the response of Leydig cells to TMCM. Values are the mean of 4-5 replicate wells and are expressed as percent of control. Arrows indicate a significant increase or decrease in androgen release in that experiment. Letters in the 'ENZ. for MØ' and 'ENZ. for L¢' represent a particular batch of type I collagenase. Vertical lines emphasize the changes in enzyme preparation used in the experiment.

However, the results indicate that the collagenase preparation may have a significant impact on the effect of TMCM on Leydig cell steroidogenesis. For instance, when TMCM were prepared with either collagenase batches 'a' or 'b', 24 h basal androgen release (after 24 h in culture) was increased in all but one experiment in TMCM-treated Leydig cells. In contrast, the use of collagenase batches 'c' and 'e' was accompanied by an inhibition of 24 h basal androgen release in TMCM-treated cells. These data are indicative of a possible relationship between collagenase preparation and TMCM effect on Leydig cells but should be kept in mind when designing further experiments. Macrophages are well known for their ability to respond to changes in their environment, chemical or physical, and the variability in the composition of collagenase batches could lead to variability in the release of bioactive substances by isolated macrophages.

Effect of Concentration of TMCM in the Culture Medium on Androgen Release

The TMCM that was prepared with collagenase batch 'c' (see Table 8) resulted in an inhibitory effect on 24 h basal androgen release and in either no effect or an inhibitory effect on 4 h LH-stimulated androgen release. These preparations of TMCM were also used to examine the effect of TMCM concentration on the response of Leydig cells. The results from these two experiments (Figure 33 for 24 h basal and Figure 34 for 4 h basal and LH-stimulated

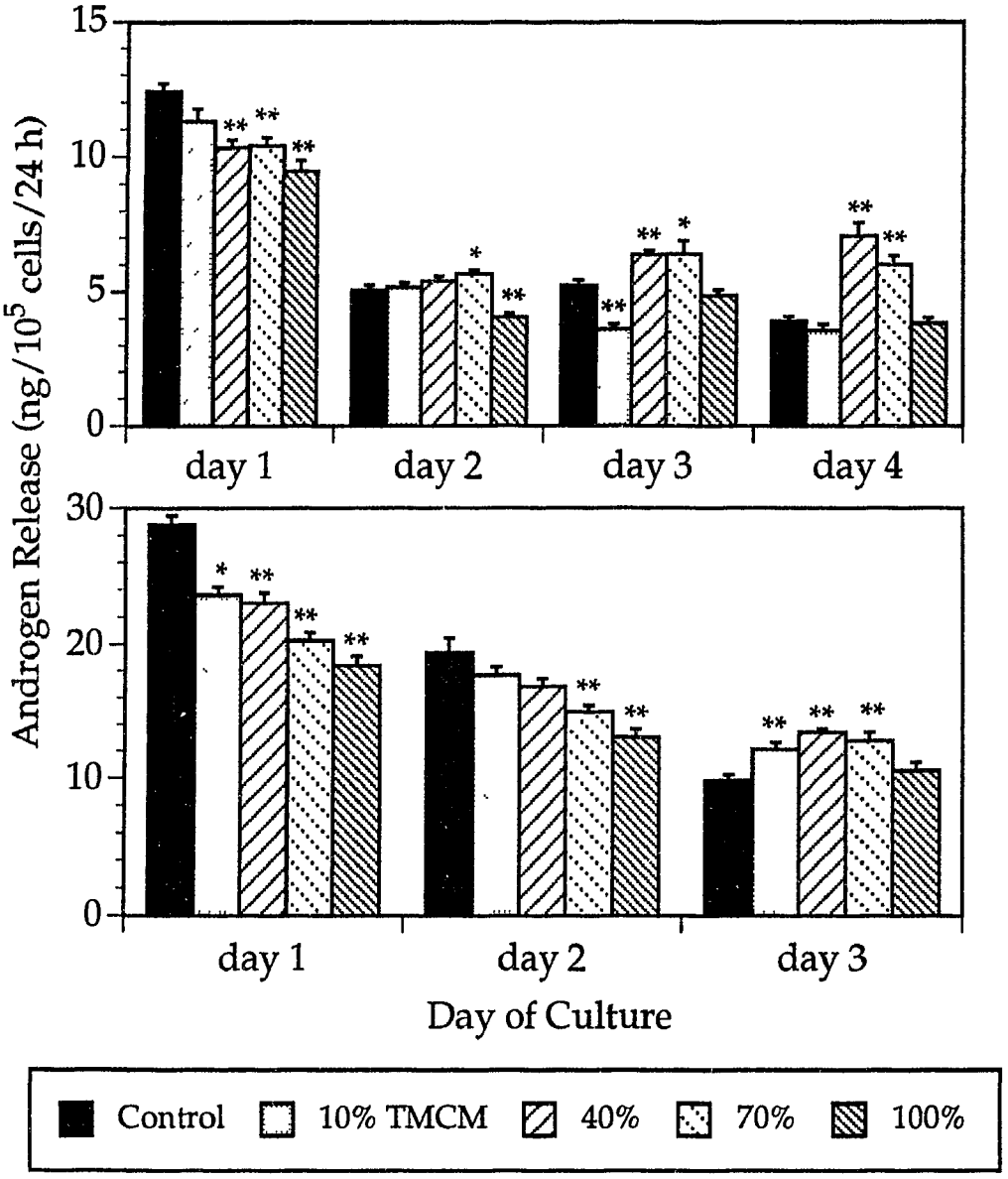

Figure 33.

Effect of percentage of TMCM on 24 h basal androgen release by Leydig cells. Shown here are two separate experiments where TMCM constituted 0% to 100% of the culture medium. Values are the means ± SEM of 8 (top panel) or 6 (bottom panel) replicate wells. *, $p<0.05$; **, $p<0.01$ by AOV followed by two-sided Dunnett t-tests.

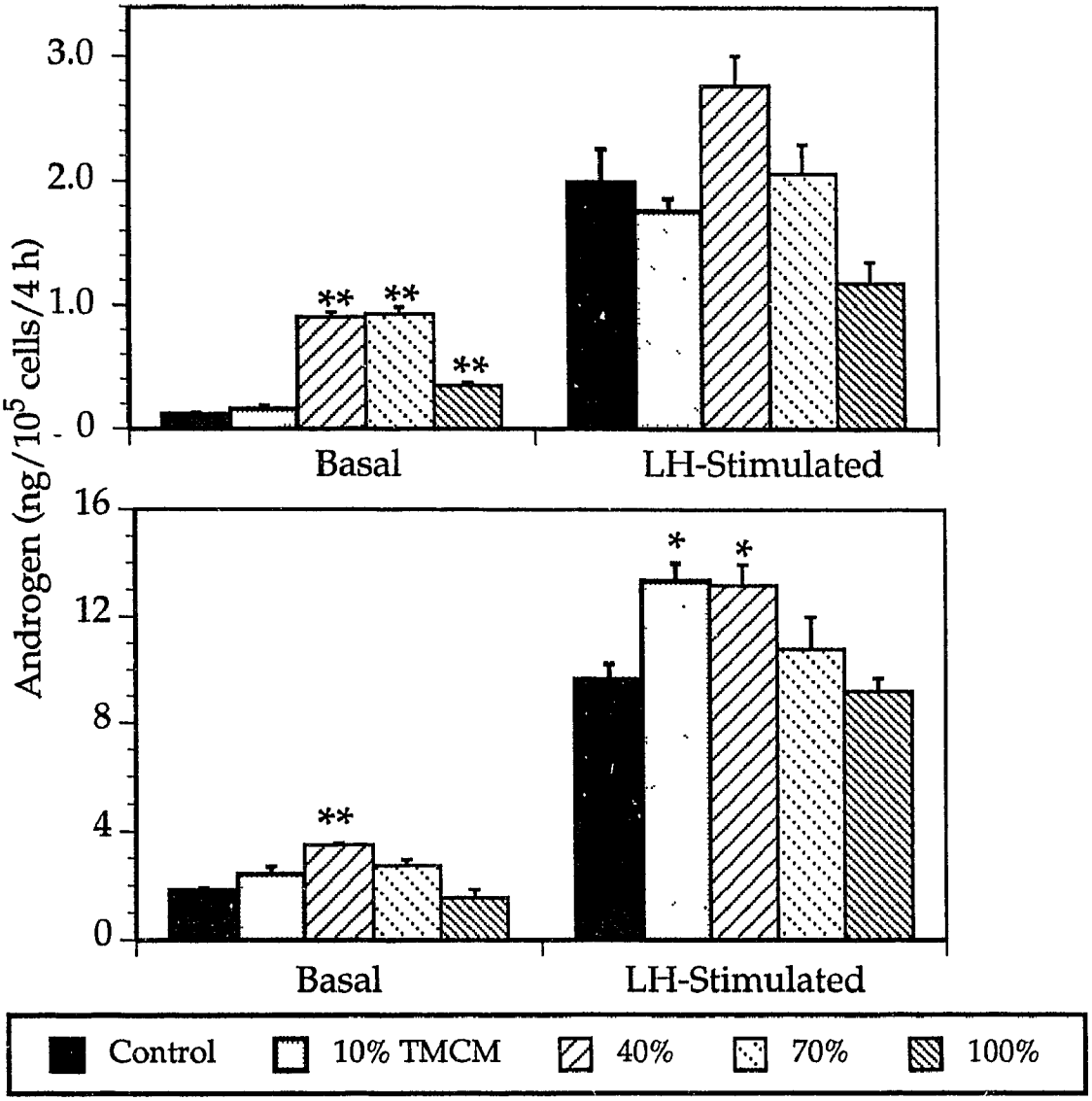

Figure 34.

Effect of percentage of TMCM in the culture medium on 4 h basal and LH-stimulated androgen release by Leydig cells. Shown here are two separate experiments where TMCM constituted 0% to 100% of the culture medium. Values are the mean ± SEM of 4 (top panel) or 3 (bottom panel) replicate wells. *, $p<0.05$; **, $p<0.01$ by AOV followed by two-sided Dunnett t-test.

androgen release) show that there is a marked effect of TMCM concentration on androgen release. In both cases low concentrations of TMCM had a stimulatory effect on basal and LH-stimulated androgen release, at least in the later part of the cultures, while 100% TMCM was either less stimulatory or frankly inhibitory. These suggest that the concentration of the bioactive substance(s) present in the TMCM may be variable with a low amount being stimulatory and a high amount being inhibitory.

Effect of Treating Testicular Macrophages with FSH on the Action of TMCM on Leydig Cell Steroidogenesis

Work by Yee and Hutson indicates (Yee & Hutson, 1983; 1985a, 1985b, 1985c) that testicular macrophages are responsive to FSH, whether it is administered *in vivo* or *in vitro*. In particular, the conditioned medium from FSH-treated macrophages is reported to have a greater stimulatory effect on basal androgen release (Yee & Hutson, 1985c). In order to verify if the latter finding could be reproduced under our culture conditions, testicular macrophages were cultured in the presence or absence of 10 ng/ml or 1 μg/ml of FSH. These concentrations were chosen because 10 ng/ml was reported to enhance the action of TMCM on Leydig cells without having, by itself, a stimulatory effect on androgen release (Yee & Hutson, 1985c) and 1 μg/ml is the concentration reported to stimulate lactate secretion and to increase amino acid incorporation into secreted protein by cultured testicular macrophages (Yee & Hutson,

1983, 1985a).

The effect of the conditioned media obtained in the presence of FSH was compared to that of conditioned medium obtained without FSH. Because FSH preparations also have LH-like activity, and thus could stimulate steroidogenesis by itself, Leydig cells were cultured in the presence or absence of the same concentration of FSH as used in the macrophage cultures. Leydig cells cultured in the presence of FSH constituted the control group for the TMCM from FSH-treated macrophage cultures (FSH/TMCM). Results from the two experiments (which used either 10 ng/ml or 1 μg/ml FSH) are shown.

In agreement with other experiments, 24 h basal androgen release was increase by TMCM on day 2 and day 3 of both experiments (Figure 35) but, unlike what has been reported by others (Yee & Hutson, 1985c), 10 ng FSH/ml had an effect on androgen release. Ten nanograms FSH per ml had a stimulatory effect on 24 h basal androgen release only on day 1 (2.4 fold increase) while 1 μg FSH/ml had a much greater effect on day 1 (8.2 fold) and was still stimulatory on day 2 (2.8 fold). The response to a maximally effective concentration of LH was not markedly affected by 10 ng FSH/ml but was substantially reduced in cells treated with 1 μg FSH/ml being only 7.8% of

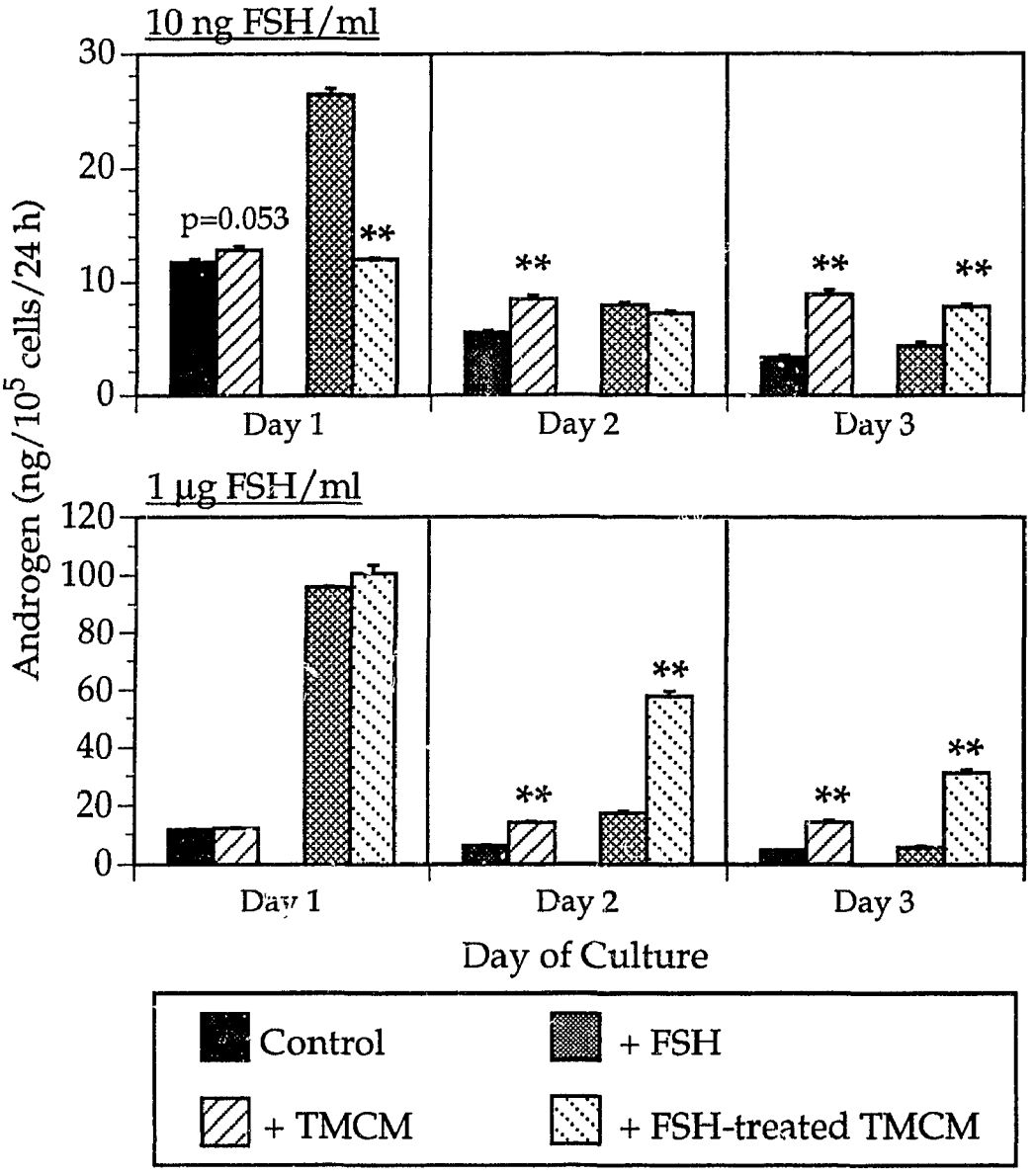

Figure 35.

Effect of treating macrophages with FSH on the action of the conditioned medium (TMCM) on 24 h basal androgen release. Top panel shows the effect of the addition of 10 ng FSH/ml to the macrophage culture while the bottom panel illustrates the effect of 1 µg FSH/ml. Comparisons were made between TMCM and Leydig cells in control medium and between TMCM from FSH-treated macrophages and Leydig cells in medium containing the same amount of FSH as used for the preparation of TMCM. Values are the mean ± SEM of 8 replicate wells. *, $p<0.05$; **, $p<0.01$ by two-tailed t-test.

that of control cells (Figure 36). The stimulatory effect of FSH alone complicates the description and interpretation of the results obtained with FSH/TMCM. The results will be described first for TMCM obtained with 10 ng FSH/ml and then for 1 μg FSH/ml.

Androgen release in response to FSH/TMCM (10 ng FSH/ml) was not markedly different from that of 'regular' TMCM. Cells treated with TMCM and FSH/TMCM released similar amounts of androgen on day 1-3 and during the 4 h test period at the end of the culture. If the response to FSH/TMCM is compared to that of cells treated with FSH alone, a somewhat different picture emerges. On day 1, FSH/TMCM had an inhibitory effect compared to the response to FSH alone. This apparent inhibitory effect could be due to less than 10 ng/ml of FSH being present in the macrophage conditionned medium due to uptake/utilization by the macrophages during the culture or due to degradation in the medium. Since 10 ng FSH/ml is only slightly stimulatory, there may not have been sufficient 'residual' FSH in the FSH/TMCM to trigger an increase in androgen release. The response to LH was the same regardless of the type of TMCM used. A 1.9-fold increase over control was induced by TMCM and a 2-fold increase was induced by FHS/TMCM (compared to FSH treated cells). There was no differences in androgen levels between TMCM- and FSH/TMCM-treated cells for both 4 h basal and 4 h LH (Figure 36; top panel).

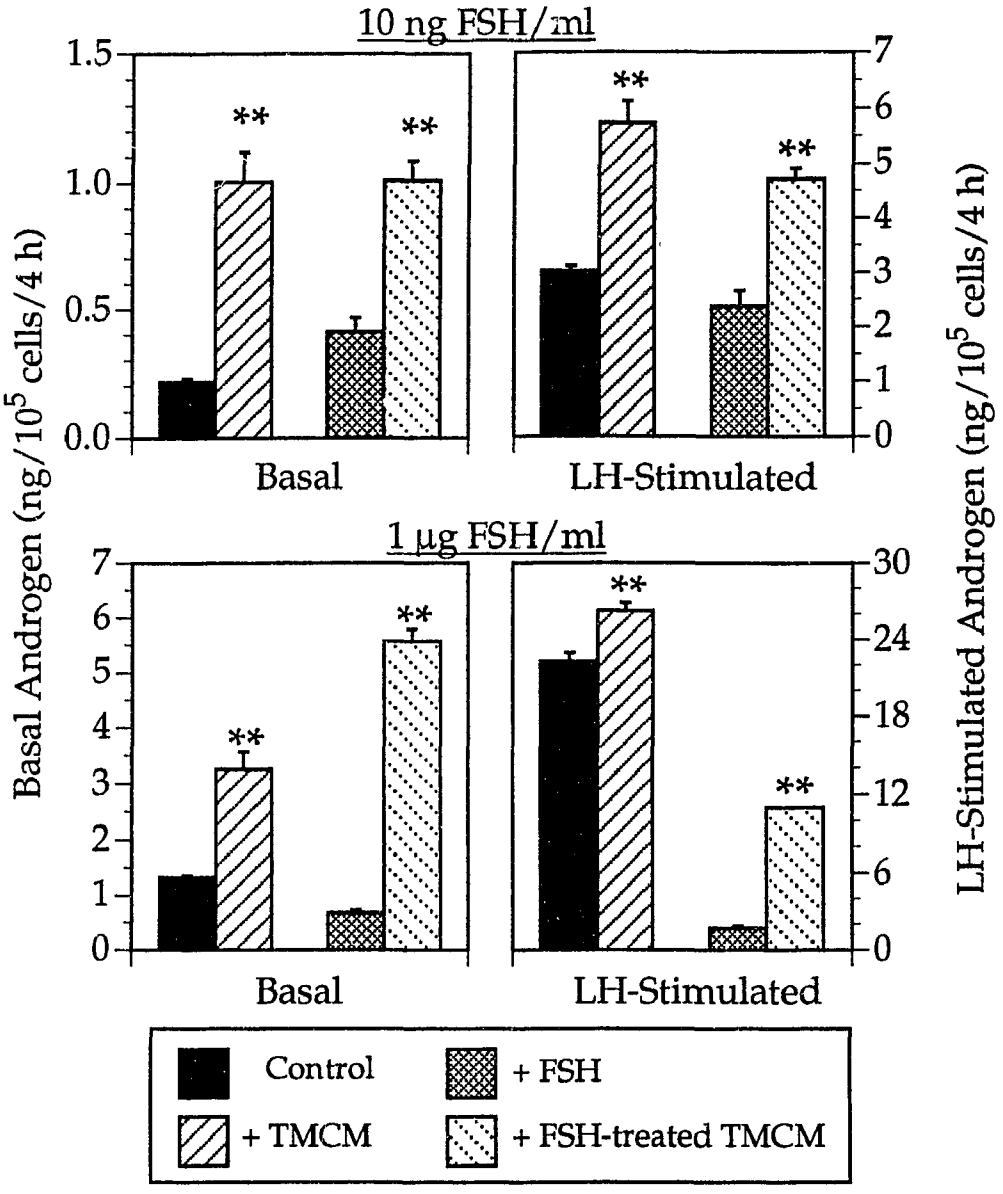

Figure 36.

Effect of preparing TMCM in the presence of FSH on 4 h basal and LH-stimulated androgen release. The top panel shows the effect of 10 ng FSH/ml and the bottom panel, the effect of 1 µg FSH/ml. Values are the mean ± SEM of 4 replicate wells. **, $p<0.01$ by two-tailed t-test between Leydig cells cultured in TMCM and Leydig cells cultured in control medium or between FSH-treated Leydig cells and Leydig cells cultured in TMCM from FSH-treated macrophages.

The effect of treating macrophages with 1 μg FSH/ml on the action of the conditioned medium is complex. Like TMCM, FSH/TMCM had a stimulatory effect on 24 h basal androgen release but androgen levels were more elevated with FSH/TMCM. The greater stimulatory effect of FSH/TMCM was present even when the response was expressed as the increase in androgen release over the appropriate control levels; the increase induced by FSH/TMCM over FSH alone is much greater than that induced by TMCM over untreated cells. FSH/TMCM induced increases of 4.8, 40.6 and 25.5 ng androgen/ml over FSH alone for day 1, 2 and 3, respectively. The increases induced by TMCM were only 0.4, 7.8 and 9.7 ng androgen/ml. Similarly, 4 h basal androgen release was increased by 4.9 ng/ml by FSH/TMCM and 1.9 ng/ml by TMCM. LH-stimulated androgen levels were 9.7 and 4 ng/ml greater with FSH/TMCM and TMCM, respectively, compared to their control group. These results suggest that treating macrophages with 1 μg FSH/ml during the preparation of the conditioned medium may enhance the stimulatory effect of such medium on basal and LH-stimulated androgen release.

Testicular Macrophage-Leydig Cell Co-cultures

Co-culturing testicular macrophages and Leydig cells together resulted in small and inconsistent changes in androgen release. Basal androgen release (Table 9) was almost unchanged during the first and second 24 h of culture.

	Exp. #	Control	CoCulture	% of Control	Control + serum or LPS	CoCulture + serum or LPS	% of Control
Day 1	1	22.4 ± .8	22.1 ± .7	98.7	–	–	–
	2	16.9 ± .7	17.5 ± .5	103.6	–	–	–
	3 (serum)	15.4 ± .4	14.3 ± .3	92.9	17.5 ± .4	14.8 ± .4 **	84.6
	4 (LPS)	8.2 ± .2	7.2 ± .1 *	87.8	8.3 ± .3	8.0 ± .2	96.4
Day 2	1	13.5 ± .8	14.6 ± .4	108.1	–	–	–
	2	14.3 ± .8	16.8 ± .7 *	117.5	–	–	–
	3 (serum)	10.6 ± .2	12.4 ± .3 *	117.0	13.0 ± .6	11.7 ± .6 *	90.0
	4 (LPS)	4.3 ± .7	4.0 ± .2	93.0	4.6 ± .2	4.7 ± .2	102.2
Day 3	3 (serum)	7.8 ± .5	8.6 ± .3	110.3	4.6 ± .4	5.8± .4 p=0.057	126.1

Table 9.

Effect of coculture on 24 h basal androgen release. Macrophages were isolated as described in the Materials and Method and cultured for 24 h. Leydig cells were then plated on top of the macrophage layer and cultured for 1 to 3 days. In two experiments cells were also cultured in the presence or absence of serum (1% FBS) or LPS. Values are the means of 4 to 10 replicate wells per group. *, $p<0.05$; **, $p<0.01$ by two-tailed t-test.

On day 1 there was a small inhibitory effect in one experiment. On day 2, the co-culture had a small stimulatory effect in 2 cultures and no effect in the other two experiments. There was a stimulatory effect of the co-culture on 4 h basal androgen release after 24 h, 48 h or 72 h of culture (Table 10). This effect was small, ranging from 114.3% to 132.4% of control and reached significance in only one experiment. The effect of the co-culture on the response to LH (100 ng/ml) was very variable. In two experiments an inhibitory effect was seen (83.5% and 80.7% of control, both after 24 h of culture; only the first one reached significance). In one experiment, the co-culture was stimulatory (127.5%, after 72 h of culture) and in the fourth, it was without effect (109.4%, after 48 h of culture).

Macrophages are usually cultured in the presence of serum. An experiment was set up to see if 1% FBS would enhance the small effect of the co-culture. In this experiment (experiment #3 in Table 9 and 10), the co-culture had no effect on basal androgen release on day 1 and day 3 and during the 4 h period at the end of the culture but was stimulatory on day 2. The response to LH was slightly, but not significantly, inhibited by the co-culture. Serum alone had a stimulatory effect on Leydig cells but co-culture done in the presence of serum had an inhibitory effect on day 1 and day 2 and a stimulatory effect on day 3 (compared to Leydig cells cultured with serum). The presence of serum did not affect 4 h basal but inhibited the response to LH to a greater extent

	Exp. #	Control	CoCulture	% of Control	Control + serum or LPS	CoCulture + serum or LPS	% of Control
4h Basal	1; 24h	5.02 ± .23	5.91 ± 1.01	117.7	--	--	--
	2; 24h	4.28 ± .23	5.67 ± .30 **	132.5	--	--	--
	3; 72h (serum)	1.17 ± .04	1.40 ± .15	119.7	2.43 ± .24	2.47 ± .15	101.6
	4; 48h (LPS)	0.28 ± .02	0.32 ± .04	114.3	0.44 ± .07	0.48 ± .08	109.1
4h LH-Stimulated	1; 24h	67.86 ± 2.17	56.69 ± 2.06 **	83.5	--	--	--
	2; 24h	48.02 ± 4.02	61.25 ± 1.42 *	127.6	--	--	--
	3; 72h (serum)	19.19 ± 1.33	15.49 ± 1.35 p=0.087	80.7	24.88 ± 1.04	16.49 ± .65 **	66.3
	4; 48h (LPS)	2.45 ± .49	2.68 ± .24	109.4	2.13 ± .28	2.95 ± .18 *	138.5

Table 10.

Effect of coculture on 4 h basal and LH-stimulated androgen release. Macrophages were isolated as described in the Materials and Method and cultured for 24h. Leydig cells were then plated on top of the macrophage layer and cultured for 1 to 3 days, followed by and additional 4 h in the presence or absence of LH (100 ng/mL). The duration of the coculture is indicated next to the experiment number. In two experiments cells were also cultured in the presence or absence of serum (1% FBS) or LPS. Values are the means of 4 to 5 replicate wells per group. *, $p<0.05$; **, $p<0.01$ by two-tailed t-test.

than in co-culture without serum. While these results are only preliminary, they suggest that the use of serum may affect the results of the co-culture.

Activated macrophages release many bioactive substances and the effect of the addition of LPS (bacterial lipopolysaccharides), a known activator of macrophages, on the co-culture was assessed (experiment #4). The presence of LPS did not markedly change the effect of the co-culture on basal androgen release except that the inhibitory effect of the co-culture on day 1 seen in this experiment was abolished when LPS was used. LPS-treated co-culture had a small stimulatory effect on the response to LH, while the co-culture done without LPS had no effect on LH-stimulated androgen release. The lack of a marked effect of LPS could be due to the fact that isolated testicular macrophages are probably already activated as a result of the isolation procedure.

DISCUSSION

For years, androgen release by Leydig cells was believed to be under the almost exclusive control of LH and the generation of intracellular cAMP. It is now widely accepted that Leydig cells can respond to numerous extra-pituitary factors and that cAMP is not the only second messenger system involved in the regulation of Leydig cell steroidogenesis. Much remains to be learned about the physiological importance of these findings but there is no doubt that what will emerge is a model of the regulation of Leydig cell steroidogenesis that is far more complex than first thought and encompasses mechanisms that allow for the fine tuning of Leydig cell function at the local level.

Originally thought to be a factor with functions restricted to the immune system, IL-1 has now been shown to affect a wide range of non-immune cells and systems. It is often described as the link between the immune and endocrine system but much evidence also supports its production at the local level, probably independently of the immune system. Investigations into the effects of IL-1 on Leydig cell function were originally motivated by the presence of resident macrophages in the interstitial space of the testis, their close association with Leydig cells and the fact that IL-1 is a major product of macrophages and monocytes. Currently, there is little evidence supporting or refuting the release of IL-1 by testicular macrophages. There is, however, some

evidence that Sertoli cells release an IL-1-like substance which could play a role in the paracrine regulation of Leydig cell function. The possible impact of IL-1 released in the periphery on Leydig cell function has received little attention.

The work presented here indicates that IL-1 can influence Leydig cell steroidogenesis *in vitro* and thus could play a role in the *in vivo* regulation of steroidogenesis in adult rat Leydig cells. The discussion of the results will first deal with the observed effects of IL-1 on androgen levels and with the site of action of IL-1 in the steroidogenic pathway. This will be followed by a look at the observations relating to the mode of action of IL-1, particularly the second messenger system(s) that is (are) involved. The effects of testicular macrophages conditioned medium on Leydig cell steroidogenesis will be discussed next. Finally, the physiological importance of these findings will be addressed.

Effect of IL-1α on Basal Androgen Release by Leydig Cells.

Basal androgen release was enhanced in cells treated with IL-1α. This effect was detectable after 24 h in the presence of IL-1α and increased with the duration of the treatment. The maximum stimulatory effect of IL-1α was observed on the third day of treatment and was fully reversible. While the stimulatory action of IL-1α on basal androgen release occured with great consistency there is some controversy in the literature regarding this effect of

IL-1. In agreement with the findings presented here, Verhoeven et al. (1988) reported a stimulary effect of IL-1 on basal androgen release by Leydig cells from prepubertal rats. The effect occured, however, within a few hours of the onset of treatment while in the present study there was usually a 20 - 24 h latency period. Warren et al. (1990) also reported an increase in basal androgen release with unpurified adult rat testicular cells. On the other hand, Calkins et al. (1988, 1990b) reported inconsistent changes in basal testosterone levels. In addition, they found high doses of IL-1 to be inhibitory and low IL-1 doses stimulatory, an observation the data presented in this thesis does not support. Similarly, Fauser et al. (1989) failed to observe any IL-1-induced changes in basal androgen release by neonatal rat Leydig cells.

It is difficult to assess the reason(s) for such inconsistency in results between the various groups but a few differences in protocols may be involved. It is unlikely that the different forms of IL-1 used are the cause of the differences between reports. Calkins et al. (1988) and Verhoeven et al. (1988) both used purified human IL-1β and yet obtained different results. Fauser et al. (1989) using a recombinant human IL-1β did not see a stimulatory effect while Warren et al. (1990) also using recombinant IL-1β and we, using a recombinant human IL-1α did see such an effect. IL-1α and IL-1β share only limited amino acid homology (26%), but they usually bind to the same receptor (Dinarello et al. 1989) and induce similar biological response in a wide variety

of cells (Dinarello, 1988a,b). In the present work, IL-1β was found to be more potent than IL-1α but both forms of IL-1 induced the same stimulatory effect on basal and inhibitory effect on LH-stimulated androgen release. In addition, the effect of IL-1α from a different supplier (Collaborative Research) was compared to the effect of IL-1α from Hoffman-LaRoche, the preparation used in all experiments; both preparations of IL-1α induced the same response by Leydig cells. Fauser et al. (1989) also report that IL-1α and IL-1β induce similar effects in Leydig cells but that IL-1β appears to have greater activity. Calkins et al. (1990b) looked at the effects of different types and sources of IL-1 and also concluded that IL-1β is more potent that IL-1α but that both induced similar effects, which did not include an enhancement of basal androgen release. In their hands, human recombinant IL-1α was 100 times less potent than recombinant human IL-1β and recombinant mouse IL-1α. It is surprising that Leydig cells would show such a difference in sensitivity to human IL-1α and mouse IL-1α since there is a high degree of homology between species for a given form of IL-1 (Nishida et al., 1989). A weaker activity for IL-1α has been reported with other systems (Scapigliati et al. 1989; Xiao & Levine, 1986) and differential effects of IL-1α and IL-1β have also been reported (Chin et al., 1987; Katsuura et al., 1988). The IL-1-like activity identified in the testes appears to be of the α and not the β type but this IL-1-like substance has not yet been fully characterized (Khan et al., 1987).

Other possible causes for the differences in the response to IL-1 are the different ages, source and/or lineage of the rats from which Leydig cells were purified. Fauser et al. (1989) used neonatal rats, Verhoeven et al. (1988) used prepubertal rats while this study and that of the two other groups used adult rats. Rats were all obtained from different suppliers. In particular it is conceivable that neonatal Leydig cells may have a different response to IL-1 than more mature cells.

The inclusion of serum in the culture media used by Calkins et al. (1988) may be one factor which may explain the difference between their results and the results from the present study. Many IL-1 inhibitors have been identified in biological fluids, including serum (Larrick, 1989). However, other effects of IL-1 on Leydig cells (for example the inhibitory action on gonadotropin-stimulated androgen release) were not blocked and thus IL-1 inhibitors may not be a key factor. The use of serum may, however have an indirect effect on the results. Contaminating cells such as macrophages may interfere with the action of IL-1. Neonatal and prepubertal testis contain few macrophages (Hutson, 1990) but adult rat interstitial space contains a substantial macrophage population and Leydig cell preparation from adult rat are contaminated with macrophages. The Leydig cell preparations used in the experiments presented in this thesis contained 7-9% macrophages at the time of plating. Calkins et al. (1988, 1990a, 1990b) do not specify the proportion of macrophages in their

preparation but it is likely to be similar to the preparation used in the current work since the purification methods were comparable. However, because of their use of serum it is possible that contaminating macrophages attach and/or survive better in their cultures than in ours. Macrophages are responsive to IL-1 (Dinarello, 1985) as well as many other substances, including steroids (Vernon-Roberts, 1969) and may release other factors that interfere with the stimulatory effect of IL-1 on basal androgen release. In addition, peritoneal macrophages can metabolize steroids (Milewich et al., 1982) and thus, if testicular macrophages also possess that ability, their presence in the culture could affect the androgen levels in the conditionned medium. All of these possibilities are, of course, speculations and it is, at this time, very difficult to reach a better understanding of these differences in results. A new procedure that allow for the preparation of Leydig cells virtually free of macrophages (Dirami, Poulter & Cooke, 1991) should be useful in determining if contaminating macrophages play a role in the variability of the reported effects of IL-1 on Leydig cells.

IL-1 has a mitogenic effect on a number of cell types, including T-cells, B-cells, fibroblasts (Dinarello, 1985; Oppenheim et al., 1986), thyrocytes (Kawabe et al., 1989; Yamashita et al., 1989) and granulosa cells (Mori et al., 1989). Under normal conditions, Leydig cells do not divide *in vivo* or *in vitro* but the possibility that the increase in basal androgen levels was the result of

a mitogenic effect of IL-1 on Leydig cells was explored. In this study the DNA content of the cultures was not affected by 3 days in the presence of IL-1α. Furthermore, Verhoeven et al. (1988), who have also reported increased basal androgen release with IL-1, expressed the release of steroid relative to the protein content of the culture. Therefore, had Leydig cell proliferation been the cause of the increase in basal androgen release, this method of expressing the results would have corrected for the increase in cells number and no increase in basal androgen release would have been seen.

The causes of the time-related decline in androgen release in adult Leydig cell cultures are not fully understood. The major drop in androgen release that occurs on the second day of culture has been attributed to the loss of cells that have poorly attached and/or the death of cells damaged during the isolation procedure. It does not appear that IL-1 increases basal androgen release by enhancing cells attachment or survival. This is supported by the absence of a difference in DNA content as well as by the results of experiments where IL-1 was added only after the initial drop in androgen release had occured.

It is interesting that a 24 h IL-1 treatment has a long lasting, although completely reversible stimulatory effect on basal androgen release. While it is possible that some IL-1 may have remained in the culture dish — no special

procedures, other than extra washing of the plates were used to remove residual IL-1 — it may also be that IL-1 induces changes in Leydig cells that have long lasting effects.

Inhibitory Effect of IL-1 on LH-stimulated Androgen Release

Treatment with IL-1 inhibited the stimulatory effect of LH on androgen release. This effect, in contrast to the effect basal androgen release, is a consistent finding in studies using both immature and adult Leydig cells although the reported latency period varies from 6 to 48 h (Calkins et al., 1988; Fauser et al., 1989; Verhoeven et al., 1988). In the present study, the inhibitory effect increased with the duration of exposure to IL-1α and, in most cases, became significant only after 3 days of treatment. Again, the difference in potency between the different sources and types of IL-1 used, and/or the use of immature Leydig cells may explain some of these differences in latency. Other cases of long latency periods in the appearance of an effect of IL-1 have been reported (Suda et al., 1989). This is consistent with the induction of gene transcription and protein synthesis often associated with IL-1 treatment (Ashizawa et al. 1989; Horuk & McCubrey, 1989; Razet al. 1988; Suda et al., 1989).

Verhoeven et al. (1988) described the effect of IL-1 on LH-stimulated androgen release by prepubertal Leydig cells as biphasic. That is, IL-1 was

found to enhance the response of Leydig cells to low concentration of LH but to inhibit the response to high LH concentrations. These results are in agreement with the findings in this report. The present work shows that IL-1α-treated cells released more androgen in response to concentrations of less than 1 ng LH/ml than untreated cells. However, when 10 or 100 ng LH/ml were used, IL-1α-treated cells released significantly less androgen than control cells. Because of the large difference in basal androgen release between the treated and untreated groups and because of the significant statistical interaction between IL-1α treatment and LH concentration the data was transformed to express the response to LH as the 'increase in androgen release over basal'. This transformation of the data revealed that treating cells with IL-1α decreased the response to all concentrations of LH. This inhibition was small with low concentrations of LH but was much more dramatic as the concentration increased. This finding could be extended to the other stimulatory agents used (dbcAMP or pregnenolone) at most of the concentrations tested. Verhoeven et al. (1988) have also shown that the stimulatory effect of IL-1α was additive or nearly additive to that of other agonists such as LHRH, EGF and Sertoli Cell Factor (SCF). Their observations are an indication that the stimulatory effect of IL-1α is mediated by a mechanism that is different from the one used by LHRH, EGF and SCF. A similar additive effect between IL-1 and submaximal concentration of LH has been observed and may indicate that the stimulatory effect of IL-1 can be observed as long as the concomitant inhi-

bitory effect of IL-1 does not become rate limiting (Verhoeven et al., 1988). Fauser et al. (1989) did not observe a biphasic effect of IL-1 on the response of neonatal cells to increasing concentration of LH. However, they also did not observe the stimulatory effect of IL-1 on basal androgen release.

Site of Action of IL-1

There is very little information regarding the site of the stimulatory action of IL-1. The present study and that of Verhoeven et al. (1988) show that under basal conditions IL-1 causes an increase in both C19 and C21 steroids thus suggesting that IL-1 stimulates steroidogenesis at a step prior to progesterone formation. Inhibition of the transformation of added pregnenolone by IL-1 (present study) as well as that of progesterone and 17α-hydroxyprogesterone (Fauser et al., 1989; Verhoeven et al., 1988) are indicative that IL-1 exerts an inhibitory effect on the 17α-hydroxylase/C_{17-20} lyase. This conclusion is further supported by the increase in the levels of C21 steroids that accompanies the inhibition of C19 steroids release (Fauser et al., 1989; Verhoeven et al., 1988). IL-1 thus appear to have two sites of action: a stimulatory effect at a step prior to the formation of C21 steroids and an inhibitory effect at the later step leading to the formation of C19 steroids from C21 precursors. As Verhoeven et al. (1988) have suggested, the stimulatory effect of IL-1 on steroidogenesis would be observed as long as the increased amount of available progesterone does not exceeds the capacity of the inhibited

17α-hydroxylase/C_{17-20} lyase to metabolize it into androgens. Anakwe and Payne (1987) have presented evidence that the levels of 17α-hydroxylase/C_{17-20} lyase activity must be reduced by 90% before it becomes rate limiting. If such a dramatic decrease in enzyme activity must occur before the inhibitory effect of IL-1 can be detected, it could explain why, in some case, the inhibitory effect of IL-1 requires a long lag period and increases with time (this report; Fauser et al., 1989). Measuring the level of 17α-hydroxylase/C_{17-20} lyase activity throughout the treatment period may provide some insight on this problem.

Another mechanism by which IL-1 could modify the levels of androgens is by modulating their conversion into dihydrotestosterone or estrogen. There is little information regarding this possibility except that Verhoeven et al. (1988) used a 5α-reductase inhibitor to prevent the metabolism of testosterone to DHT and yet observed both the stimulatory effect on basal and the inhibitory effect on LH-stimulated androgen release. Whether IL-1 affects the conversion of testosterone to estrogens has not been examined.

Mode of action of IL-1

1 - The IL-1 Receptor

In the culture system used in this study, maximization and maintenance of the effect of IL-1α on both basal and LH-stimulated androgen production

required that IL-1α be present continuously in the culture media. Removal of IL-1α resulted in the gradual return of 24 h basal androgen levels to control values and in a marked reduction of the inhibition of LH-induced androgen release. This observation is interesting because it is not consistent with the rapid down-regulation of the IL-1 receptor demonstrated in many cells types (Dinarello, 1988b). However, as discussed in the introduction, there are several types of IL-1 receptors and their properties differ. Recent research, for example, has shown that IL-1 receptors from the Raji cell line, unlike those on EL-4 cells, do not down-regulate even after 24 h of exposure to IL-1 (Horuk & McCubrey, 1989). Evidence indicates that there are two main types of IL-1 receptors, one being associated with B-cells, the other with T-cells (Bomsztyk et al., 1989; Chizzonite et al., 1989). The 'B-cell' IL-1 receptor is the one that does not appear to down regulate. Both receptors have now been cloned (Mcmahan et al., 1991; Sims et al., 1988). An IL-1 receptor has not yet been identified on rat Leydig cells but the mouse testis has been shown to contain IL-1 receptors (Takao et al., 1990). Further characterization should reveal which 'category' the testicular IL-1 receptors belong to.

2 - Mode of Action of IL-1 in Leydig Cells

The mode of action of IL-1α has been elusive and is still the focus of much controversy. Several second messengers have been proposed and some may be active in some cell types but not others (Chedid, Shirakawa, Naylor &

Mizel, 1989; Mills et al. 1990; Mochan et al., 1989; Muñoz et al. 1990 Shirakawa et al., 1988; Zawalich & Zawalich, 1989). Rapid increases in cAMP levels have been described in some cell types while phospholipase C (PLC) may be active in the same or other cells (Shirakawa et al., 1988; Muñoz et al., 1990; Turunen, Mattila & Renkonen, 1990). The rapid phosphorylation of cytosolic proteins in fibroblasts (Bird & Saklatvala, 1989; Kaur & Saklatvala, 1988) and the EGF receptor in leukocytes (Matsushima et al., 1988) following IL-1 treatment is also documented. Protein kinase A (PKA) (Turunen, Mattila & Renkonen, 1990) and protein kinase C (PKC) (Gottlieb et al., 1989; Muñoz et al., 1990) activation appear to play a role in several cells types but a recent report by O'Neill et al. (1990) proposes that a novel, G-protein-linked kinase may be responsible for the phosphorylation of cellular proteins in T-cells (EL-4 cell line). Despite the lack of a thorough understanding of the mode of action of IL-1, it is becoming increasingly evident that IL-1 is able to trigger several signal transduction systems and that in cell types where more than one system is activated, these systems interact to produce the final biological response (Mochan et al., 1989; Muñoz et al., 1990)

a) Cyclic AMP as Second Messenger

Coupling of the IL-1 receptor to adenylate cyclase has been proposed, and mediation of the action of IL-1 by cAMP has been demonstrated for several cell types (Chedid et al., 1989; Shirakawa et al., 1988). In Leydig cells,

changes in cAMP levels which paralleled the changes in androgen levels; basal cAMP levels were increased while LH-stimulated levels were inhibited, all after 3 days of treatment (this report). However, there was no changes in cAMP levels during the first 4 h of exposure to IL-1 and hardly any changes after 48 h with IL-1. Inhibition of hCG-stimulated cAMP release by IL-1 has been previously described (Calkins et al., 1988) but, to my knowledge, the present report is the first showing an increase in basal cAMP release with IL-1 treatment. It should be mentioned, however, that others (Calkins et al., 1988) have measured cAMP levels after 24 h of IL-1 treatment, a time at which time the present work also shows no effect of IL-1α on both basal and LH-induced cAMP levels. Verhoeven et al. (1988) whose results on androgen release are very similar to the results presented here, did not see any changes in cAMP levels.

The absence of a change in cAMP levels after 4 h and 52 h in the presence of IL-1 (this report) casts a doubt on the involvement of cAMP as the primary mediator of IL-1 action in Leydig cells. In cell systems where IL-1 is believed to act primarily through the activation of adenylate cyclase, cAMP levels increase within 10-20 minutes after the introduction of IL-1 (Chedid et al., 1989; Shirakawa et al., 1988). While the present findings do not rule out the possibility that cAMP may be involved in the modulation of androgen release by IL-1, they suggest that changes in cAMP levels may be the consequence of

the activation by IL-1 of some other second messenger system. For instance, work by Mochan et al. (1989) suggest that in human synovial cells, changes in cAMP levels after IL-1 treatment are secondary to the activation of PLA_2 and prostaglandin production rather than a primary event (Mochan et al., 1989). A similar observation has been made in adrenal cells where a rise in PGE_2 levels precedes and appear essential for the increase in IL-1-induced rise in cAMP levels (Tominaga et al., 1990). Although Calkins et al. (1988) have demonstrated a marked increase in PGE_2 levels in IL-1β-treated Leydig cells, the inhibitory effect of IL-1 on LH/hCG-induced androgen release could not be prevented by blocking the rise in PGE_2 with a cyclooxygenase inhibitor.

There is good evidence that IL-1 exerts an inhibitory effect at a step distal to activation of adenylate cyclase. IL-1 inhibits the response to cAMP analogs (this report, Calkins et al., 1988; Fauser et al., 1989), forskolin (Calkins et al., 1988; Fauser et al., 1989) and cholera toxin (Calkins et al., 1990a). In addition and as previously mentionned, IL-1 inhibits the conversion of 25-OH-cholesterol, pregnenolone, progesterone or 17α-OH-progesterone into androgens indicationg that the site of the inhibition is probably the 17α-hydroxylase/C_{17-20} lyase enzyme. A small decrease in hCG binding has been reported by Calkins et al. (1988) but in light of the evidence towards a 'post-adenylate cyclase' site of action, the importance of this finding is unclear. None of the other studies on Leydig cells have dealt with the effect of IL-1 on

LH/hCG binding. IL-1 inhibits LH/hCG binding in granulosa cells (Gottshall et al., 1987; Mori et al., 1989) but the mode of action of IL-1 in these cells may be different from that in Leydig cells since IL-1 failed to inhibit dbcAMP-induced progesterone formation in granulosa cells (Mori et al., 1989). The site of action of IL-1 in granulosa cells appears to affect mostly LH/hCG receptor binding and adenylate cyclase activation.

b) Involvement of a PTX-Sensitive G-protein

A pertussis toxin-sensitive G-protein has been implicated in the signal transduction mechanism for IL-1 in some (Chedid et al., 1989; Crawford & Crossley, 1989; O'Neill et al., 1990) but not all systems (Mills et al., 1990) and an inhibitory G-protein is present in Leydig cells (Platts, Schulster & Cooke, 1988; Vihko & Huhtaniemi, 1989). The possibility that the inhibitory effect of IL-1α on LH-stimulated androgen release and cAMP levels may be mediated by an inhibitory G-protein was investigated. In agreement with previous reports (Platts, Schulster & Cooke, 1988; Vihko & Huhtaniemi, 1989), it was found that basal and LH-stimulated cAMP levels in the spent medium from Leydig cell cultures are increased in the presence of pertussis toxin (PTX). Although PTX slightly enhanced the stimulatory effect of IL-1α on basal cAMP levels, it was unable to block the inhibitory effect of IL-1α on LH-stimulated cAMP levels and androgen release. These results suggest that a PTX-sensitive G-protein is not involved in IL-1α signal transduction in Leydig cells. It should

be kept in mind, however, that in this study, PTX was only introduced after 3 days of culture in the presence of IL-1. Since the inhibitory effect of IL-1 on LH-stimulated androgen and cAMP release is related to the duration of the treatment, PTX could have been introduced too late in the experiments to have an effect. Still, the inhibitory effect of other factors on Leydig cells have been shown to be unresponsive to PTX. Vihko and Huhtaniemi (1989) have recently reported that PTX is unable to block the inhibitory effect of a rat seminiferous epithelial factor on hCG-, cholera toxin- and forskolin-induced cAMP formation.

A direct interference with adenylate cyclase through a G_i protein is not the only mechanism by which cAMP formation can be inhibited. Receptor mediated activation of PLC involves a non-PTX sensitive G-protein and the resulting activation of PKC may lead to the inhibition of cAMP formation (Mukhopadhyay, Schumacher & Leidenberger, 1985). One should also consider the possibility of an effect on the degradation of cAMP. Experiments from the present study were not conducted in the presence of a phosphodiesterase inhibitor in order to maintain comparable conditions between experiments and thus facilitate results comparisons. This approach does not permit us, however, to rule out the rate of cAMP degradation as a factor in the observed changes.

c) Arachidonic Acid Metabolites in IL-1 Action and Leydig Cell Steroidogenesis

The finding by Verhoeven et al. (1988) that the stimulatory effect of IL-1 is additive to those of LHRH, EGF, AVP and SCF suggests that these agonists activate a different second messenger cascade from IL-1. The mode of action of these factors is believed to involve the hydrolysis of membrane phospholipid, the activation of PKC and/or the generation of arachidonic acid (AA) metabolites. It is interesting that IL-1, LHRH, EGF and SCF all induced similar changes in Leydig cell steroidogenesis; a stimulation of basal androgen release accompanied by an inhibition of the response to LH/hCG. This contrasts with the additivity of the effect of IL-1 and that of other agonists which would suggest that they use a similar signalling mechanism. IL-1 could possibly increase the action of other agonists through its well documented stimulatory effect on the levels and/or activity of enzymes such as PLA_2 and cyclooxygenase for example (Burch & Tiffany, 1989; Kerr et al., 1989). An interesting mechanism of action of IL-1 has been proposed to explain the requirement by Jurkat T cells for two different signals for optimal production of IL-2 (Didier et al., 1988). The first signal induces phosphatidylinositol hydrolysis and the formation of IP_3 and DAG but inhibits phosphatidylserine (PS) synthesis, an essential cofactor of PKC activation. This first signal appears to induce insufficient PKC activation and a second signal is required for IL-2 (interleukin-2) synthesis. The second signal can be IL-1 or a direct activator of PKC.

IL-1 is believed to stimulate PS synthesis thus allowing sufficient PKC activation to trigger IL-2 production. Such a complex mechanism of action may not exist in Leydig cells but this theory emphasizes the complexity of the mode of action of IL-1 and its ability to interact with other second messenger systems.

Results from this study indicate that products of the lipoxygenase pathway of AA metabolism are involved in the stimulatory effect of IL-1α on Leydig cell steroidogenesis. Blocking the lipoxygenase pathway with NDGA nearly completely abolished the rise in basal androgen release induced by IL-1α but did not affect the inhibitory action of IL-1α on LH-stimulated androgen levels. The DNA content of the cultures indicate that cell number was not affected by NDGA but this does not necessarily imply that the cells are alive. Consequently, other tests of cell viability such as trypan blue incorporation (Peterson & Stulbery, 1964) or, as suggested by Rommert, Molenaar & van der Molen (1985), diaphorase activity should be performed in future studies to confirm that the inhibitory effect of NDGA on the response to IL-1α and LH is not caused by a cytotoxic effect on Leydig cells. This study also provides additional evidence for a role of the lipoxygenase pathway in the stimulation of Leydig cell steroidogenesis by LH. Several reports have demonstrated a role of the lipoxygenase pathway in Leydig cell steroidogenesis (see below). Nevertheless, our results on the role of this pathway in the

response of Leydig cells to IL-1 should be interpreted with caution since only one lipoxygenase inhibitor was used. An effect(s) of NDGA non-related to its action on the lipoxygenase enzymes could play a role in the response seen in our experiments. For example, the antioxidative nature of the NDGA molecule (Needleman et al., 1986) could play a role in the early stimulatory effect of NDGA. The use of other lipoxygenase inhibitors (see Rainsford, 1988 for review) should permit to confirm the results obtained with NDGA. Results with both indomethacin and ASA indicates that the metabolism of AA through the cyclooxygenase pathway does not play a role in Leydig cell steroidogenesis *in vitro*.

i) Arachidonic Acid Release and Metabolism in Leydig Cells

Several groups have demonstrated that AA or its metabolites play a role in the regulation of Leydig cell steroidogenesis. Increasing the levels of free intracellular AA by using PLC (Didolkar & Sundaram, 1987, 1989), phorbol myristate acetate (PMA) (Didolkar & Sundaram, 1987) or by adding AA to the incubation medium (Didolkar & Sundaram, 1987, 1989; Lin, 1985) augment basal and sub-maximally stimulated androgen release but inhibits maximally stimulated androgen release by Leydig cells (Abayasekara, Band & Cooke, 1990; Didolkar & Sundaram, 1987). This effect is strikingly similar to that induced by IL-1. Conflicting results have been reported when phospholipase A_2 (PLA_2) is used to raise the levels of free AA. Some groups report similar

results as those described above (Abayasekara, Band & Cooke, 1990) but others have failed to see any changes in basal or LHRH-stimulated androgen release (Didolkar & Sundaram, 1987). Similarly, no clear picture has emerged from the use of inhibitors of PLA_2. Abayasekara, Band & Cooke (1990) have recently reported that dexamethasone (that stimulates production of lipocortin which inhibit PLA_2), quinacrine (that stabilizes lysosomes and prevents phospholipid hydrolysis by PLA_2) and p-bromophenacyl bromide (pBPB) (that binds the histidine residue on PLA_2 and reduces Ca^{++} binding and thus PLA_2 activation), which are all inhibitors of PLA_2 but which act through different mechanisms, have no effect on basal but inhibit maximum LH-stimulated androgen release. Others have shown that PLA_2 inhibitors inhibit basal as well as LHRH-stimulated androgen release (Didolkar & Sundaram, 1989; Lin, 1985). All groups used similar concentrations of PLA_2 inhibitors but differences in incubation protocols may explain the different effects on basal androgen release. It is interesting to note that chloroquin and quinacrine, which are both lysosomal stabilizing agents, inhibit LHRH-induced androgen release in addition to AA- and LH-stimulated androgen release (Didolkar & Sundaram, 1989). This indicates that their action may extend beyond the inhibition of PLA_2 and the release of AA (Abayasekara, Band & Cooke, 1990).

It is somewhat paradoxical that in the presence of maximally effective concentrations of LH/hCG, androgen release is inhibited not only by increasing

AA (Abayasekara, Band & Cooke, 1990; Didolkar & Sundaram, 1987) but also by inhibiting the release of AA with PLA_2 inhibitors (Abayasekara, Band & Cooke, 1990). It has been proposed that the amount of free AA in the cell may be important in determining the direction of the androgen response and this may provide an explanation for this apparent contradiction in findings. Didolkar & Sundaram (1987) have shown that low concentration (12.5 μM) of exogenous AA enhances hCG-induced testosterone secretion but higher concentrations (up to 100 μM) are inhibitory. Similar results have been obtained with various concentrations of PLC and PMA. It may be that LH/hCG induces the release of low levels of AA which are essential for the full androgen response. Blocking PLA_2 and thus the release of AA would therefore inhibit the androgen response to LH/hCG. The addition of exogenous AA may bring the level of AA to a high enough concentration to result in an inhibition of the response. This biphasic effect of AA was not seen for basal testosterone release which was increased with all concentration of AA (Didolkar & Sundaram, 1989).

Most, but not all reports indicate that AA must first be metabolized in order to modulate Leydig cell steroidogenesis. AA can be metabolized through three different pathways; the cyclooxygenase pathway which processes arachidonic acid to prostaglandins and thromboxanes; the epoxygenase pathway which leads to the production of epoxygenated acid while the

pathway produces leukotrienes, hydro(per)-oxy fatty acids and free radicals (Rainsford, 1988; Smith, 1989). Schematic representations of the main pathways of arachidonic acid release and metabolism and of the site of action of the various inhibitors can be seen in Figures 37 and Figure 38.

When all three pathways are blocked with the inhibitor ETYA, the stimulatory effect of PMA, PLC (Didolkar & Sundaram, 1987), and exogenous AA (Didolkar & Sundaram, 1987; Didolkar & Sundaram, 1989) is abolished. ETYA also inhibits LHRH (Didolkar & Sundaram, 1989), dbcAMP (Dix, Habberfield, Sullivan & Cooke, 1984) and LH-stimulated (Dix, Habberfield, Sullivan & Cooke, 1984; Didolkar & Sundaram, 1987) androgen release. Unless ETYA exerts some effects unrelated to AA metabolism, these finding support a role for AA metabolism not only in LHRH action but also in that of LH and the activation of PKA by cAMP. These results also indicate that AA itself is inactive and must be metabolized before exerting its effect on Leydig cell steroidogenesis. They do not indicate, however, which of the three pathways is/are involved.

ii) Involvement of Cyclooxygenase Metabolites in the Mode of Action of IL-1 in Leydig Cells

In vivo LH treatment is followed by an increase in *in vitro* $PGF_2\alpha$ production by Leydig cells (Haor et al., 1979) and *in vitro* LHRH (Molcho,

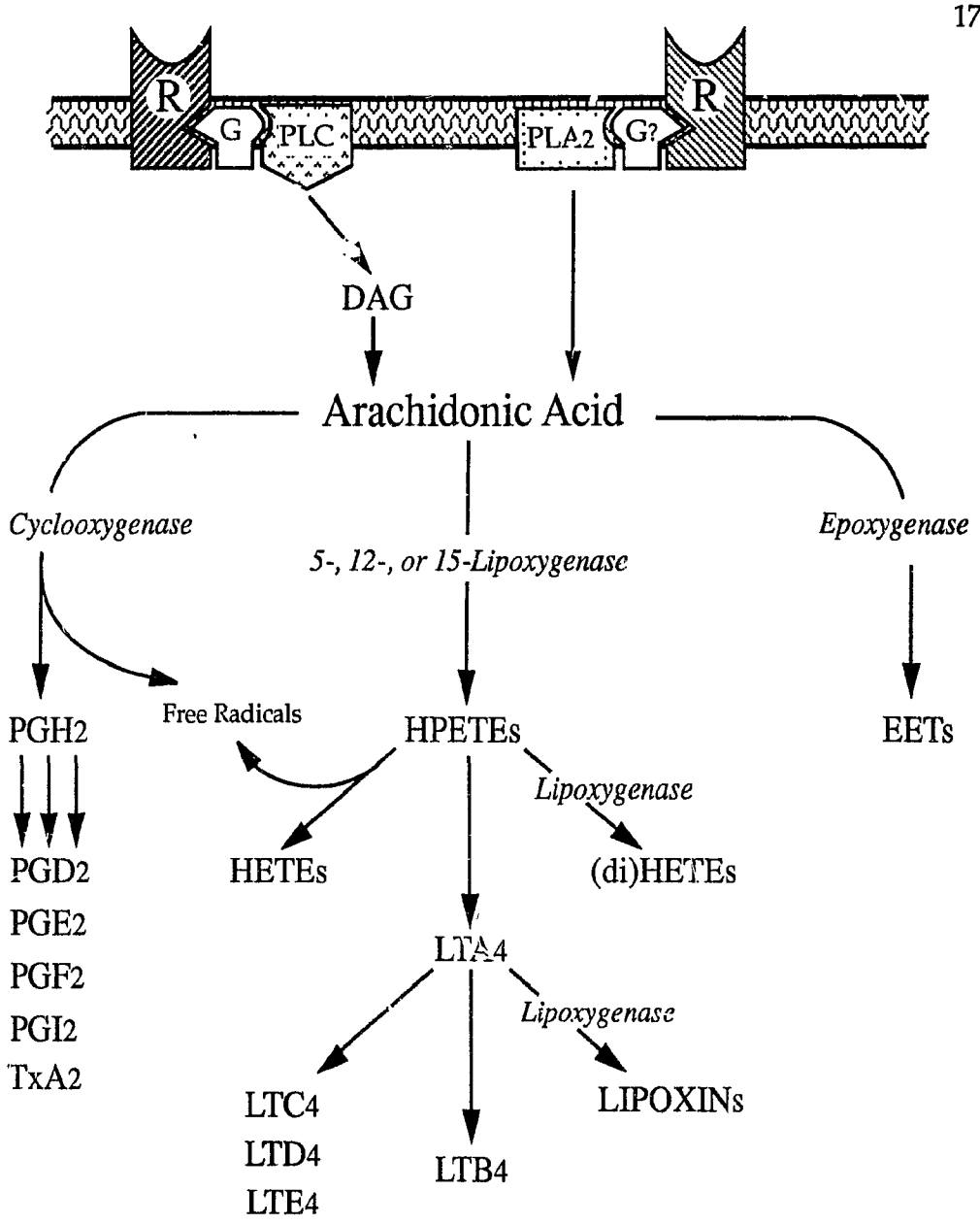

Figure 37.

Pathway leading to the release of arachidonic acid and the formation of eicosanoids. Only key intermediates, metabolites and enzymes are shown. Adapted from Smith, 1989 and Rainsford, 1988.

DAG = Diacylglycerol
(di)HETE = Dihydroxyeicosatetraenoic acid
EET = Epoxyeicosatrienoic acid
G = G protein
HETE = Hydroxyeicosatetraenoic acid
HPETE = Hydroperoxyeicosatetraenoic acid
LT = Leukotriene
PG = Prostaglandin
PLA2 = Phospholipase A2
PLC = Phospholipase C
R = Receptor
Tx = Thromboxane

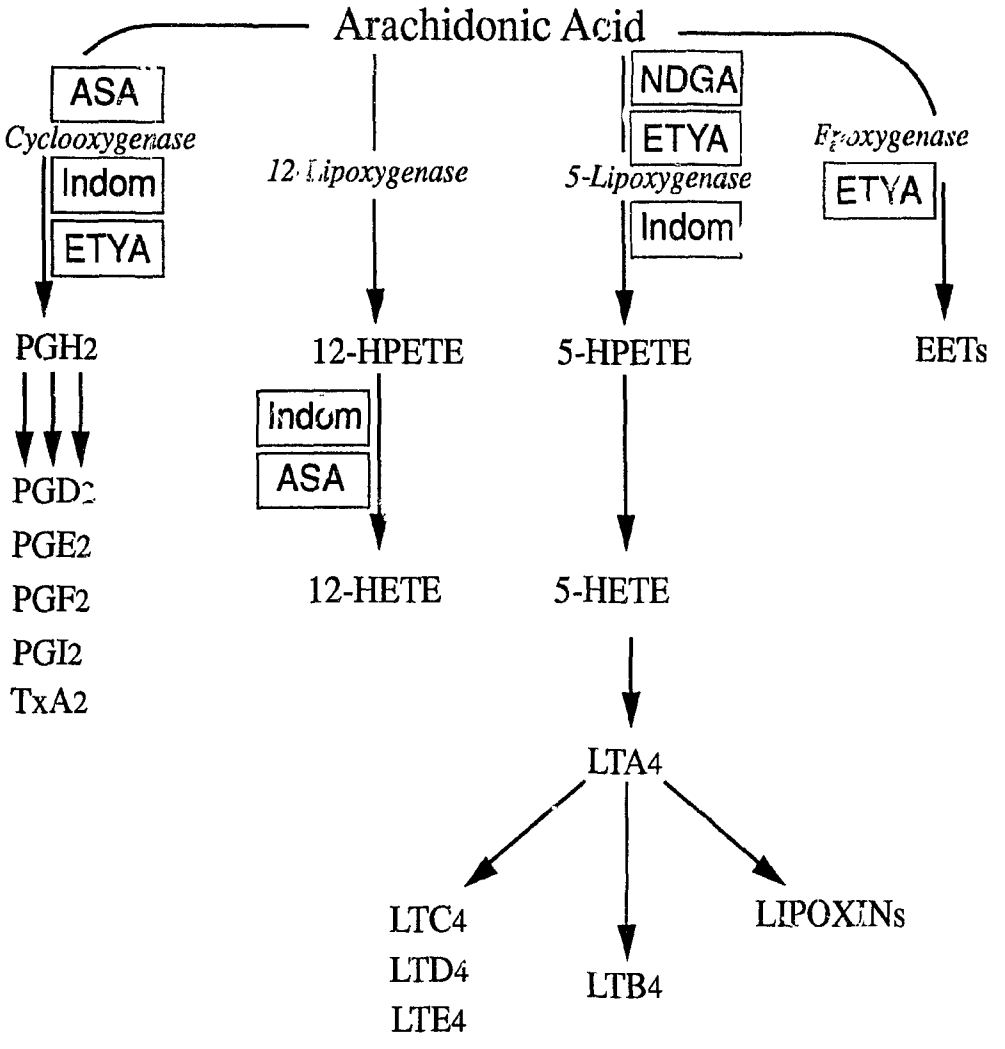

Figure 38.

Site of action of inhibitors of arachidonic acid metabolism. Only relevant steps are illustrated here. Inhibitors appear in boxes.
Adapted from Smith, 1989

ASA = Acetylsalicylic acid
ETYA = 5,8,11,14-eicosatetrayonic acid
Indom = Indomethacin
NDGA = Nordihydroguaiaretic acid

Other abbreviations are defined in Figure 37

1984a & 1984b) and IL-1 (Calkins et al., 1988) also cause an increase in PGE_2 production. Despite these findings, the cyclooxygenase pathway does not appear to regulate *in vitro* Leydig cell steroidogenesis since blocking it has no effect on androgen production (this report; Dix et al., 1984; Lin, 1985). There have been a few reports showing that indomethacin enhances basal and LHRH- or sub-maximally LH-stimulated androgen release (Sullivan, Abayasekara & Cooke, 1988; Lin, 1985; Didolkar & Sundaram, 1989). However, at the concentration used in these studies, indomethacin may loose its specificity for the cyclooxygenase pathway and also inhibit other pathways of AA metabolism (Rainsford, 1988). The effect of 20 μM indomethacin, a concentration believed to specifically inhibit cyclooxygenase, and of 100 μM indomethacin were compared and it was found that they elicit completely different responses. ASA (400 μM), which is also a cyclooxygenase inhibitor, and 20 μM indomethacin were without effect when used alone or in conjunction with IL-1α and/or LH. In contrast, 100 μM indomethacin caused a significant increase in basal androgen release while significantly inhibiting LH-stimulated androgen release. This response was in many ways similar to that obtained with the lipoxygenase inhibitor NDGA thus suggesting that the effect of 100 μM indomethacin could be attributed to an inhibition of the lipoxygenase pathway (Rainsford, 1988). The apparent lack of effect of cyclooxygenase inhibitors on androgen release does not imply that prostaglandins and/or thromboxanes are without effect in testicular function.

It has been suggested, although results are still inconclusive, that the increase in $PGF_2\alpha$ that follow *in vivo* hCG treatment may be involved in the increase in interstitial fluid volume that also follows hCG treatment (Abayasekara et al., 1988).

iii) The Role of Lipoxygenase Metabolites in the Mode of Action of IL-1 in Leydig Cells

There is substantial evidence that the lipoxygenase pathway of arachidonic metabolism is involved in Leydig cell steroidogenesis. In short term incubations, the lipoxygenase inhibitor NDGA significantly enhances basal androgen production, but inhibits LH-stimulated androgen production (Didolkar & Sundaram, 1987, 1989; Dix, Habberfield, Sullivan & Cooke, 1984; Lin, 1985). While these findings are in agreement with results presented in this thesis, the long term culture system used in here has revealed that the stimulatory effect of NDGA is short lived and significant only during the first 24 h of culture. The reason for the stimulatory effect of NDGA on basal androgen release is unclear. In freshly isolated cells, the levels of free AA may still be elevated as a result of the influence of hormones or other signal molecules from their *in vivo* environment. In the absence of further stimulation, available free AA is depleted and the stimulatory effect of NDGA disappears. It would be interesting to see if delaying the addition of NDGA by 24 h has an influence on the stimulatory effect of NDGA on basal androgen release. Results

obtained with general inhibitors of AA metabolism and specific inhibitors of the cyclooxygenase and lipoxygenase pathways have prompted the hypothesis that the third pathway of AA metabolism, the epoxygenase pathway, may be responsible for the stimulatory effect of lipoxygenase inhibitors. Blocking the lipoxygenase pathway would result in more AA being available for processing through the epoxygenase pathway (Didolkar & Sundaram, 1989).

Dix et al. (1984) report that NDGA not only inhibits LH-stimulated androgen release but also dbcAMP-stimulated androgen and pregnenolone production and LH-induced cAMP production. Thus, the inhibitory effect of NDGA appears to lie both at the level of cAMP formation and at a site distal to cAMP formation but prior to the conversion of cholesterol to pregnenolone. The inhibitory effect of NDGA on both androgen and progesterone production in LH-stimulated cultured Leydig cells presented here is in agreement with the findings by Dix and his coworkers (1984).

Tumour Leydig cells metabolize ^{14}C-labelled AA into leukotriene B4 (LTB4), 5-HETE and 12-HETE, a process which is inhibited by NDGA (Dix et al., 1984). In purified Leydig cells, 5-HPETE has been shown to enhance submaximally stimulated androgen release but not basal or maximally stimulated androgen production and to partially reverse the inhibitory effect of lipoxygenase inhibitors (RG5901, NDGA) and PLA_2 inhibitor (mepacrine)

(Sullivan, Abayasekara & Cooke, 1988). The *in vitro* release of an other lipoxygenase product, LTB4, is enhanced by *in vivo* hCG/LH. The effect occurs with a 4-6 hr latency period and is thought to be the result of the trophic effect of hCG/LH on lipoxygenase enzymes rather than the acute release of AA by PLA_2. Together with the absence of consistent increase in LTB4 with *in vitro* LH treatment (Sullivan, Abayasekara & Cooke, 1988) these finding suggest that LTB4 may not have an acute effect on *in vitro* steroidogenesis.

Solano, Dada & Podesta (1988) have recently reported that the lipoxygenase pathway may play a central role in adrenal steroidogenesis. Their findings indicate that lipoxygenase metabolites may be a common intermediate for both the cAMP-dependent and cAMP-independent stimulation of adrenal steroidogenesis by ACTH and angiotensin II, respectively, in a cell-free system. In Leydig cells, NDGA also blocks the stimulatory effect of agents operating through a cAMP-dependent (LH) and cAMP-independent pathways (LHRH) (Didolkar & Sundaram, 1989; Lin, 1985). Inhibitors of AA release and metabolism also block the release of ACTH-induced aldosterone release by glomerulosa cells (see Solano, Dada & Podesta, 1988). These findings suggest that lipoxygenase metabolites play a key role in both adrenal and gonadal steroidogenesis. Others, however, have reported that it is the cyclooxygenase pathway that mediates the effect of IL-1 on adrenal cells (Tominaga et al., 1990).

In summary, metabolites of AA have been shown to play a role in Leydig cells steroidogenesis. The lipoxygenase pathway appears to be an important component of the stimulatory effect of LH and LHRH and may be a common element of the signalling pathway(s) of these molecules. Furthermore, lipoxygenase products may play a similar role in adrenal steroidogenesis. Epoxygenase products may have a stimulatory effect on basal androgen release by Leydig cells although evidence for this is indirect. The cyclooxygenase pathway, despite having been shown to be active in Leydig cells, does not seem to play a role in the acute regulation of Leydig cell steroid production.

The stimulatory effect of IL-1 on the production of AA metabolites by numerous cell types is well documented. Products of the cyclooxygenase pathway, for example PGE_2 and PGI_2, are believed to mediate at least some of the effects of IL-1 on monocytes/macrophages, muscle cells, fibroblasts, synovial cells, astrocytes, chondrocytes, osteoblasts, endothelial cells (see Dinarello, 1986 and Martin & Resch, 1988 for reviews), pituitary cells (Bernton et al., 1987), thyrocytes (Kawabe, et al., 1989), the hypothalamus (Navarra et al., 1990), adrenocortical cells (Winter, et al., 1990; Tominaga et al., 1990) and juxtaglomerular cells (Antonipillai, Wang & Horton, 1990). The production of lipoxygenase metabolites such as 5-and 12-HETE and LTB4 can also be increased by IL-1 and may mediate the effects of IL-1 on chondrocytes (Kerr,

et al., 1989), smooth muscle cells (Mugridge et al., 1989) and adrenal glomerulosa cells (Natarajan et al., 1989).

iv) The Effect of IL-1 on Arachidonic Acid Release and Metabolism

IL-1 stimulates eicosanoid production through several mechanisms. The increase in AA metabolites following IL-1 exposure occurs rapidly in some cases while in others a substantial lag period is observed. Rapid changes in eicosanoid levels is probably the result of IL-1-induced AA release. How IL-1 triggers this release is still unclear and the mechanism may vary with the cell type. Some evidence indicates that IL-1 may stimulates the hydrolysis of membrane phopholipids via PLC, thus resulting in the production of diacylglycerol (DAG) from which AA could be released through the action of a DAG lipase (Smith, 1989). Depending on the cell type, IL-1 treatment results in the hydrolysis of phosphatidylcholine in Jurkat cells (Rosoff, Savage & Dinarello, 1988), phosphatidylethanolamine in mesengial cells (Kester et al., 1988) or phophatidylinositol in macrophages (Wijelath et al., 1988), synovial cells (Mochan et al., 1989) and pancreatic cells (Zawalich & Zawalich, 1989). Phospholipid hydrolysis is not, however, present in all cell types (Abraham et al., 1987; Didier et al.,1988; Mills et al., 1990; Rosoff et al.,1988).

It has also been suggested that IL-1 stimulates the activity and/or production of various enzymes involved in the production of eicosanoids.

Increased PLA_2 and/or cyclooxygenase activity has been demonstrated in fibroblasts (Burch & Tiffany, 1989; Raz et al., 1988), chondrocytes (Kerr et al., 1989; Suffys, van Roy & Fiers, 1988) and synovial cells (Mochan et al., 1989). It is suggested that IL-1 enhances the activity of those enzymes by inducing gene expression. Increased PLA_2 mRNA levels have been shown to accompany the increase in PLA_2 activity in chondrocytes (Kerr et al., 1989). Furthermore, there is often a delay between IL-1 treatment and increased eicosanoid levels which can be blocked by protein synthesis inhibitors (Kerr et al., 1989; Burch & Tiffany, 1989). Burch & Tiffany (1989) have recently shown that IL-1 treatment not only increases PGE_2 levels in 3T3 fibroblasts but also greatly enhances their response to bradykinin. In normal kidney cortex fibroblasts and normal synovicytes, bradykinin has little effect on PGE_2 levels. In contrast, in experimental hydronephrosis and in rheumatoid arthritis those cells show a strong PGE_2 response to bradykinin. It is postulated that macrophages invading the diseased tissue may release IL-1 and/or TNF which then cause the enhance response to bradykinin (see Burch & Tiffany, 1989). How this effect is brought about is not resolved yet but either increased transcription of genes involved in AA metabolism and/or increased availability of free AA could be occuring. A better understanding of the relationship between IL-1 and the eicosanoids may be shed some light on the mechanisms involved in abnormal tissue response.

The data in this thesis suggests that, as shown for other cells types (Mochan et al., 1989; Rosoff, Savage & Dinarello, 1988; Shirakawa et al., 1988), there may be more than one mechanism of IL-1α signal transduction in Leydig cells. The stimulatory effect of IL-1α on basal androgen release can be blocked by inhibiting the lipoxygenase pathway of AA with NDGA indicating that IL-1α stimulates the production of lipoxygenase metabolites either by enhancing the release of AA and/or by increasing the production and/or activity of lipoxygenase enzymes. On the other hand, the inhibitory effect of IL-1α on LH-stimulated androgen release is not blocked by NDGA and thus could be mediated by a separate mechanism. It should be kept in mind, however, that NDGA also had an inhibitory effect on LH-stimulated androgen release. The present work and that of others (Fauser et al., 1989; Verhoeven et al., 1988) demonstrate that the inhibitory action of IL-1α is located at the level of the 17α-hydroxylase/C_{17-20} lyase. On the other hand, the lipoxygenase pathway is believed to enhance either cholesterol synthesis and/or transport and/or cholesterol side-chain cleavage (Dix et al., 1984). Blocking the lipoxygenase pathway would therefore inhibit androgen release at a step prior to that inhibited by IL-1α. As a result, the intrinsic inhibitory effect of NDGA on LH-induced androgen release may mask its effect on the IL-1-induced inhibition of LH-stimulated androgen release. Measuring the androgen release in the presence of exogenous pregnenolone or progesterone in Leydig cells treated with both IL-1 and NDGA may indicate whether the inhibitory effect of IL-1

on 17α-hydroxylase/C_{17-20} lyase activity is altered by NDGA.

d) Other Mechanisms of Action of IL-1 in Leydig Cells

If IL-1α does not inhibit LH-stimulated androgen release through an action on the lipoxygenase pathway then an alternative mechanism could involve the gradual desensitization of Leydig cells due to the stimulatory effect of IL-1α on either or both adenylate cyclase and PKC activity. The present work shows that the inhibitory effect of IL-1α on LH-stimulated androgen release increases with the duration of exposure to IL-1α (this report). Adult Leydig cells are well known to lose their ability to respond to LH with sustained exposure to stimulatory agents such as cAMP (Georgiou, Perkins & Payne, 1987) or activated PKC (Dufau, 1988). Because the levels of basal cAMP are increased with IL-1α (this report) and the activation of PKC by Il-1 has not been ruled out, it is conceivable that long-term exposure to IL-1α may result in the desensitization of Leydig cells to LH. While this hypothesis has not been disproved, the rapid onset of the inhibitory effect of IL-1 shown by others does not support it (Calkins et al., 1988; Verhoeven et al., 1988).

Effect of IL-1 on Other Steroidogenic Cells

IL-1 has been shown to influence the function of other steroid producing cells. The presence of macrophages in the corpus luteum of mice (Kirsch et al., 1981), the well documented interaction between gonadal steroids and the

immune system (see Grossman, 1985 for review), and the dramatic rise in serum IL-1 levels during the luteal phase of the menstrual cycle in women (Cannon & Dinarello, 1985) were a few of the findings that prompted investigations into the effect of IL-1 on ovarian function. There is now much *in vitro* and *in vivo* indications that IL-1 is a significant modulator of ovarian physiology. IL-1 has been shown to inhibit basal and LH-stimulated progesterone production by medium size porcine follicles as well as cAMP accumulation and LH/hCG binding (Fukuoka et al., 1988, 1989; Mori et al., 1989). IL-1 also inhibits the luteinization of porcine granulosa cells (Fukuoka et al., 1988; Mori et al., 1989) and FSH-induced increases in LH receptors and progesterone release in rat and porcine granulosa cells *in vitro* (Gottschall et al., 1987; Mori et al. 1989) as well as FSH-induced estrogen release by granulosa cells from small size porcine follicles (Mori et al., 1989) and rat granulosa cell (Gottshall, Katsurra & Arimura, 1989). The mode of action of IL-1 in granulosa cell seems to involve mostly LH/hCG receptor binding and interference with adenylate cyclase activation. The absence of an effect on dbcAMP-induced progesterone formation indicates that IL-1 does not affect post-cAMP formation events in the granulosa cells (Mori et al., 1989). An other observation that contrasts with results from Leydig cell cultures is that IL-1 stimulates granulosa cell proliferation. It should be noted, however, that this effect was obtained in the presence of substantial amounts of serum (10%), conditions that have not been used with Leydig cells. Rivier and Vale (1989) have recently added conside-

rable credibility to the postulated role of IL-1 on gonadal function by showing that intraperitoneal injections of IL-1 to hypophysectomized female rats results in an inhibition of gonadotropin-induced estrogen and progesterone release *in vivo*. They have also shown that the inhibition of LH release observed in rats receiving IL-1 directly into the lateral cerebral ventricle is likely mediated by an effect on the hypothalamus rather than by a decreased sensitivity of the pituitary to GnRH. Bernton et al. (1987) have demonstrated a direct stimulatory effect of IL-1 on pituitary cells in culture. Such an effect may, however, be highly dependent on culture conditions and the sex and state of the animal from which they are obtained (Lumpkin, 1987). It is nevertheless clear that IL-1 can exert both a direct and an indirect effect on gonadal function.

Adrenal steroidogenesis is also influence by IL-1 but it is still unclear whether IL-1 has a direct effect on the adrenal cortex or whether its action is mediated by the hypothalamus and/or the pituitary. There is little doubt that IL-1 has a marked stimulatory effect on the release of CRF by the hypothalamus (Berkenbosch et al., 1987; Sapolsky et al., 1987) and many feel that this is the main site of action of IL-1. A direct effect on the release of ACTH by the anterior pituitary has been demonstrated by some (Bernton et al., 1987; Suda et al., 1989) but not others (Berkenbosch et al., 1987; Sapolsky et al., 1987). The first indication of a possible direct effect of IL-1 on the adrenals came from the work of Roh et al. (1987) who showed that corticosteroid output by

perfused adrenal glands was increased by IL-1. More recently, Winter and coworkers (1990) and Tominaga & Arimura (1990) have found that IL-1 increases corticosteroid output by mixed bovine and rat adrenocortical cells. On the other hand, Gwosdow, Kumar & Bode (1990) have shown no effect of IL-1 with adrenal organ culture, a finding that supported the a lack of effect of IL-1 on Y-1 adrenal tumor cell reported by Woloski et al. (1985). However, Gwosdow's group has recently indicated that IL-1, contrary to their earlier report (Gwosdow, Kumar & Bode, 1990), may exert a stimulatory effect on adrenal organ cultures (Gwosdow, Spence & Bode, 1990).

The stimulatory effect of IL-1 has been shown by most to involve the release of prostaglandin(s) (Roh et al., 1987; Tominaga & Arimura, 1990; Winter et al., 1990). Winter et al. (1990) have proposed that IL-1 stimulates the release of prostaglandins by a subpopulation of adrenal cells and that, in turn, those prostaglandins stimulate the release of corticosteroids by the steroidogenic cells. IL-1 would then be part of a paracrine mechanism regulating adrenocortical function.

Effect of TMCM on Leydig Cell Steroidogenesis

The examination of the effect of IL-1 on Leydig cell function was based on the assumption that IL-1 or IL-1-like molecules could be found in the vicinity of Leydig cells. The most obvious source of IL-1 appeared to be the

population of macrophages that reside in the interstitial space of the testis. Consequently, the effect of testicular macrophages on Leydig cell function was also studied. The data obtained in this project remained preliminary as more efforts were put towards the characterization of the action of IL-1 on Leydig cells. Nevertheless, the results provide information that will be usefull in the design of future studies of the effect of testicular macrophages on Leydig cell steroidogenesis.

This work has established that IL-1 has an effect on Leydig cell steroidogenesis and that conditionned medium from testicular macrophage (TMCM) can also modulate androgen release. However, as will be described below, the effect of TMCM and IL-1 differ noticeably. This may indicate either that testicular macrophages do not release IL-1 in culture or that they release other substances as well; the effect seen would then be the result of the combined action(s) of all these substances. These possibilities will be further discussed below.

1 - Effect of TMCM on Androgen Release

Culturing Leydig cells in the TMCM resulted in an enhancement of both basal and LH-stimulated androgen release. TMCM-treated cultures released the same amounts of androgen over 3 consecutive 24 h periods while androgen release in control cultures decreased gradually but markedly over the same

period of time. After 72 h in the presence of TMCM, the response to LH (100 ng/ml for 4 h) was increased compared to control. The effect of TMCM was time-dependent as no significant differences in basal and LH-stimulated androgen release was detected after 24 h of treatment. Yee and Hudson (1985c) reported similar results although the onset of the effect of the TMCM was much more rapid. Culture conditions and/or cell density in Yee and Hutson's experiments may have induced the release of different amounts and/or types of bioactive molecules which might play a role in the rapidity at which changes are observed. It is interesting that, similarly to the effect of TMCM, the effect of IL-1α is also time-dependent. In addition, the lag phase in the action of IL-1 also varies markedly between the reports from various research groups.

2 - Effect of Culture Conditions on TMCM Action

One of the difficulties associated with the study of tissue macrophage function is related to the method used for their isolation. Macrophages are cells 'designed' to respond to changes in their environment and they have the potential to release a large number of different bioactive products (Nathan, 1987). Since the process of isolating testicular macrophages involves disturbing this environment it is likely that their activity *in vitro* will differ from their 'normal', *in vivo* behaviour (Meltzer, 1981). In the system used in this project, the use of collagenase to disperse the testicular cells probably exerts a significant effect on the macrophages. Collagenase preparations are contami-

nated with a variety of other enzymatic activities as well as with bacterial lipopolysaccharide (LPS), a well know activator of macrophages. Exposure to LPS has been shown, for example, to increase the release of IL-1 and TNF by macrophages (Old, 1987; Weinberg, 1981), two products which have the ability to influence Leydig cell function (this report, Calkins et al., 1988, 1990b; Fauser et al., 1989; Verhoeven et al., 1988; Warren et al., 1990).

There is substantial batch-to-batch variation in the composition of the collagenase preparations, particularly regarding the amounts of other enzymatic activities. This variability may increase the inter-experiment variability in culture conditions. The results presented here suggest that the batch-to-batch variability in collagenase preparation may be crucial to the final response of Leydig cells to TMCM. For instance, while early experiments with TMCM yielded an increase in androgen release by Leydig cells, later experiments conducted with macrophages isolated with different batches of collagenase resulted in variable Leydig cell responses, ranging from the absence of an effect to the presence of an inhibitory effect of TMCM on androgen release. While these results are somewhat anecdotal because of the low number of replicate experiments, they emphasize the need to carefully monitor all the steps of the isolation procedure and the culture conditions of macrophages and to maintain as much uniformity between experiments as possible. As described in the Materials and Methods, Leydig cells are also quite

sensitive to the collagenase preparation used for their isolation. However, in their case, the collagenase effect on cells function is more readily detected since it tends to interfere with normal steroidogenesis. Because macrophages can potentially release so many biactive products and it is not yet know which of those products testicular macrophages release into the culture medium, it is very difficult to monitor variability in its/their release. Changes in collagenase batch and/or other variations in culture conditions may exert an effect of the concentration of bioactive product(s) in the TMCM on the response of Leydig cells. Certain preparations of TMCM had an inhibitory effect on androgen release when used at 100% but had a stimulatory effect when used at lower concentrations. This could indicate that the same agent is present in all TMCM preparation but that its concentration varies depending on the culture conditions. IL-1 has been shown by some (Calkins et al., 1988) to have a biphasic effect on basal androgen release. However, IL-1 had an inhibitory effect on LH-induced androgen release at all concentrations tested which would tend to indicate that the active agent in TMCM is not IL-1 or, at least, is not only IL-1.

3 - Effect of FSH on TMCM Action

Testicular macrophages respond to FSH and they have specific FSH receptors (Yee & Hutson, 1983, 1985a, 1985b). FSH stimulates lactate production in testicular but not peritoneal macrophages (Yee & Hutson, 1983). *In vivo* (Yee

& Hutson, 1985b) and *in vitro* FSH (Yee & Hutson, 1985a) enhances amino acid and uridine incorportion into proteins and RNA, respectively. Intracellular cAMP levels are also increased with *in vitro* FSH treatment (Yee & Hutson, 1985a). In addition, the stimulatory effect of TMCM was shown to be enhanced when the macrophage cultures are treated with FSH (Yee & Hutson, 1985c). There was no effect of hCG, insulin, testosterone or estrogen on lactate production by testicular macrophages (Yee & Hutson, 1983), a finding which is somewhat surprising since hCG binding has been demonstrated for liver macrophages (Moore, 1986) and since the effect of steroid hormones on macrophages function is well documented (Vernon-Roberts, 1969). *In vivo* testosterone has been shown to be ineffective at stimulating macrophages activity when used alone but to potentiate the effect of estrogen on phagocytosis (Nichol, Vernon-Roberts & Quantock, 1965). Such a combination of steroids was not tested in the testicular macrophage study (Yee & Hutson, 1983).

Attempts at replicating the findings regarding the stimulatory effect of FSH on TMCM action yielded results that are not as definitive as those of Yee and Hutson (1985c). One of the problems that was encountered was that Leydig cells responded to FSH with an increase in androgen release, even with a concentration similar to that used by Yee and Hutson. Ten nanogram FSH/ml induced a 2-fold increase in androgen release and 1 µg FSH/ml

resulted in a 10-fold increase. The FSH preparation used in this project (NIAMDD-ovine FSH-14) had an LH contamination equivalent to 0.02 times NIH-LH-S1. This would translate in approximately the same LH activity as 0.14 ng/ml of the LH preparation used in this project (NIH-LH-B9; 0.7 times the activity of NIH-LH-S1) when 10 ng FHS/ml was used and as 14 ng LH/ml when 1 µg FSH/ml was used. These amounts of LH activity are compatible with the stimulatory effect of FSH on androgen release observed. Yee & Hutson did not report a significant direct stimulatory effect of 10 ng FSH/ml on Leydig cell steroidogenesis. It is possible that their FSH preparation (NIADDK-oFSH-16) had less LH activity than the preparation used in the current study. Despite these confounding factors, the data indicate that at 10 ng/ml, FSH does not enhance the stimulatory effect of TMCM. In fact, it was found that androgen release with TMCM obtained from FSH-treated macrophages (10 ng FSH/ml) was similar to the release obtained with 'control' TMCM for both basal and LH-stimulated androgen release. When a higher concentration of FSH was used (1 µg FSH/ml) there were some indications that the stimulatory effect of TMCM on basal androgen release was enhanced by FSH. However, because of the high LH activity present in the FSH-treated TMCM and in the FSH treatment alone, there was a marked depression of the reponse to LH after 3 days of culture in these treatment groups. Nevertheless, TMCM from FSH-treated macrophages had a marked stimulatory effect on LH-induced androgen release compared to FSH-treated Leydig cells. Thus, the data

indicates that while treating testicular macrophage cultures with FSH may alter the effect of the TMCM on Leydig cells, further studies of this phenomenon will require the use of an FSH preparation with much less LH contamination.

4 - Effect of Macrophage-Leydig Cell Co-culture on Androgen Release

Histological examination of the interstitial space of the testis has revealed that testicular macrophages are usually closely associated with Leydig cells (Bowman & Rowland, 1983; Miller, Niemi, Sharpe & Brown, 1986; Ohata, 1979). Numerous processes extend from the Leydig cell membrane and are inserted into invagination of the macrophage membrane (Kuopio & Pelliniemi, 1989; Miller, Bowman & Rowland, 1983). The physiological importance of these close membrane associations is unknown but could be involved in Leydig cell-macrophage communication. An earlier study by Kirsch et al. (1981) had looked at the potential effect of macrophages, which are found in the corpus luteum (CL) of several species (Bagavandoss et al., 1988; Bulmer, 1964; Gillim et al., 1969; Paavola, 1977), on steroid production by CL cells. They found that while the conditioned medium from macrophage cultures was without effect, co-culturing luteal cells with peritoneal macrophages or with autologous CL macrophages enhanced their progesterone production. They also found that CL macrophages were more active than their peritoneal counterpart, indicating a degree of specialization in macrophage functions. Peritoneal macrophages were also less active than testicular macrophages at stimulating Leydig cell

steroidogenesis (Yee and Hutson,1985c). Microscopical examination of macrophage-granulosa cells co-cultures revealed the presence of cell-cell contact between the two cell types. It was conclude that cell contact or, at least cell proximity, was an important factor in the stimulation of progesterone release by macrophages (Kirsh et al., 1981). Cell-cell contact and exchange of material between macrophages and fibroblasts has also been documented (Dean, Cooper & Stahl, 1988). These results, in addition to the presence of Leydig cell-macrophage interactions *in vivo*, prompted us to test the effect of co-culturing testicular macrophages and Leydig cells on androgen release. Unfortunately, the co-culture had very little effect. It is difficult to assess the cause of the absence of response but the culture conditions may not have been suitable for the establishment of inter-cellular interactions. Macrophages are usually cultured in the presence of serum (Adams & Edelson, 1981) and Kirsch et al. (1981) used 20% calf serum in their CL cell cultures. In this Leydig cells culture system serum was not used because of its effect on androgen release (Melsert & Rommerts, 1987). The use of 1% serum was attempted for the co-culture experiments but even such a low amount of serum affected androgen release. There was also no apparent effect of the presence of the macrophages, possibly because 1% serum was insufficient to support macrophage function.

5 - Known Macrophage Products and their Possible Involvement in the Effect of TMCM on Leydig Cells

There is no doubt that macrophages have the potential to affect Leydig cell steroidogenesis through the release of one or many bioactive products into their culture medium. Although there is currently no information regarding which one of the multiple possible macrophage products may be present in TMCM, several macrophage products are known to have an effect on Leydig cells and could be involved in the action of TMCM. A short overview of these products follows. This list is by no means exhaustive but, rather, a selection of some of the most likely candidates for a role in TMCM action on Leydig cells. An extensive list of macrophage secretory product has been published by Nathan (1987) and was used for the following selection.

IL-1 and TNF are well know products of macrophages and share numerous biological activities in other systems (Oppenheim et al., 1989). TNF has been detected in macrophages in rabbit CL (Bagavandoss, 1988). Both are also known to affect Leydig cell function. The effect of IL-1 has already been well described in the above discussion. The action of TNF on Leydig cells is less documented but Calkins et al. (1990b) recently reported that human recombinant TNFα has no effect alone but potentiates the inhibitory effect of IL-1 on LH-stimulated androgen release. Warren et al. (1990), on the other

hand, have found that recombinant TNFα has a stimulatory effect on both basal and LH-stimulated androgen release. It should be mentionned here that Warren's group is the only one who has documented a stimulatory effect of IL-1 on Leydig cells maximally stimulated with LH. It remains possible, however, that the stimulatory effect of TMCM on androgen release is mediated by TNF and/or IL-1.

Macrophages can release an insulin-like growth factor-1-type molecule (Rom et al., 1988). Interestingly, insulin and insulin-like growth factor 1 (IGF-1) have a stimulatory effect on both basal and LH-stimulated androgen release (Bellvé & Zheng, 1989; Bernier et al., 1986; De Mellow, Handelsman & Baxter, 1987; Lin, Blaisdell & Haskell, 1988; Majercik, Czerwiec & Puett, 1989; Moger & Murphy, 1987). Insulin was added to the Leydig cell culture medium but it is possible that it was not added is sufficient amounts to induce a maximal response. Additional insulin-like activity in the TMCM could then further increase androgen release.

TGFβ is another macrophage products with known effects on Leydig cells (Avallet et al., 1987; Lin, Blaisdell & Haskell, 1987; Fauser, Galway & Hsueh, 1988; Morera et al., 1988). However, TGFβ has an inhibitory effect on LH/hCG-induced androgen release and is therefore not likely responsible for the stimulatory effect of TMCM. A stimulatory effect of TGFβ on 3β-HSD is

also documented (Maudiuit et al., 1991) and thus its involvement in the action of TMCM cannot completely ruled out. Interferon gamma (INFγ) (Orava, Voutilainen & Vihko, 1988), and bFGF (Fauser, Baird & Hsueh, 1988) are other macrophage products with an effect on Leydig cells which could be found in TMCM. However, as TGFβ, their action appear to be mainly inhibitory.

The release of several cyclooxygenase and lipoxygenase products by macrophages is well documented (Nathan, 1987; Ogle et al., 1988; Vincent & Ziljlstra, 1986) and, as discussed, many of these may have an effect on Leydig cells function. The impact of exogenous arachidonic acid metabolites on androgen release is not very well understood but, again, they could be present in the TMCM and be involved in TMCM action.

Other macrophage secretory products may be involved mostly in the *in vivo* action of macrophages or in co-culture situations. IL-1 inhibitors (Arend et al., 1989; Larrick, 1989) could potentially counteract the effect of an IL-1-like molecule of Sertoli cell origin (Syed et al., 1988). Macrocortin (or lipomodulin), a phospholipase inhibitor could influence the production of arachidonic acid metabolites by other testicular cells, including Leydig cells (Blackwell et al., 1980). Fibronectin is a protein involved in cell adhesion, proliferation and differentiation and it could possibly be important for the organization of

interstitial cells *in vivo*. It could also be involved in co-culture conditions by providing a substrate onto which Leydig cells can attach and which would support better function. Fibronectin stimulates the release of IL-1 by macrophages (Beezhold & Lause, 1987) and another macrophage product, TGFβ can stimulates fibronectin production by other cells types and its incorporation in the extracellular matrix (Ignotz & Massagué, 1986). Macrophages can release a number of binding proteins involved in lipid transport such as apolipoprotein E (Basu et al., 1981) and lipid transfer protein (Tollefson et al., 1985). These could facilitate the availability of substrate for steroid hormone synthesis, for example. At this point in time there is no evidence toward the release of these molecules by testicular macrophages either in culture or *in vivo*. A role for any of these molecules in Leydig cell-macrophage interactions is also highly speculative. The polypeptide factors such as IL-1, TNF, IGF-1-like molecule or TGFβ for example, may offer the most readily verifiable means by which testicular macrophage modulate Leydig cell function. These molecules, when used alone, may not induce the same effect as TMCM but little is known about the effect(s) induced by a combination of two or more of these factors.

Physiological Implications of the Action of IL-1 and TMCM on Leydig Cell Steroidogenesis

There are two main physiological implications to the observed ability of macrophages and IL-1 to affect the release of testicular steroids. The first one concerns the paracrine regulation of testicular function and the second one pertains to the growing field of interactions between the immune and endocrine systems.

As discussed in the Introduction, the existence of local regulatory mechanism of testicular function is now well accepted. This thesis has provided evidence that IL-1α has both a stimulatory and an inhibitory effect on androgen release and that secretory product(s) from testicular macrophages can modulate Leydig cell steroidogenesis. Several findings point towards an active role for testicular macrophages in testicular physiology: the cellular membrane of macrophages and Leydig cells show close associations (Kuopio & Pilliniemi, 1989; Miller, Bowman & Rowland, 1983), macrophage morphology is altered after disruption of testicular function (Bergh, 1987) and macrophage concentration changes during development (Hutson, 1990). Despite these observations, the role played by testicular macrophages and IL-1 in the maintenance of normal gonadal function is still poorly understood.

As was emphasized in the Introduction, the 'need' for intratesticular

regulatory mechanism is linked to the variation in the nutritional requirements of developing sperm cells. For instance, certain stages of the spermatogenic cycle have a greater requirement for testosterone than others. This probably reflects a higher requirement of Sertoli cells for testosterone in order to provide adequate support for sperm cells at certain stages of maturation. There is evidence that an IL-1-like molecule is produced by Sertoli cells and its levels increase sharply at the time of puberty and the establishment of spermatogenesis (Syed et al., 1988). This molecule could conceivably reach the Leydig cells and modulate steroid release. Since Leydig cells are probably not exposed to maximally stimulating levels of LH in their *in vivo* environment it is difficult to assess the importance of the *in vitro* observation regarding the inhibitory effect of IL-1 on LH-stimulated androgen release. However, the stimulatory effect of IL-1 on basal and submaximally stimulated androgen release (this report; Verhoeven et al., 1988), if it occurs *in vivo* could results in an enhanced response of Leydig cells to submaximal gonadotropin stimulation and thus in greater local androgen concentration. Through the production of an IL-1-like molecule, Sertoli cells could therefore regulate local androgen concentration.

Testicular IL-1 could also exert its effect through the resident testicular macrophages. As demonstrated in this project and by the work of others (Yee & Hutson, 1985c) testicular macrophages can release substance capable of modulating Leydig cell steroidogenesis. As is seen with 'immune' IL-1 and

macrophages from other tissue, testicular IL-1 could induce gonadal macrophages to produce active molecules such as prostaglandins or TNF (Martin & Resch, 1988). In turn these substances could influence Sertoli cell function, capillary permeability or Leydig cell function. For example, $PGF_2\alpha$ has been proposed to regulate interstitial fluid volume (Abayasekara et al., 1988) and TNF may alter Leydig cell steroidogenesis (Calkins et al., 1990b; Warren et al., 1990).

The release of CRF by Leydig cells (see Fabbri, Tinajero & Dufau, 1990) provides another mechanism of interaction between Leydig cells, testicular IL-1 and/or testicular macrophages. IL-1 stimulates the release of CRF by the hypothalamus (Berkenbosch et al., 1987; Sapolsky et al., 1987; Navarra et al., 1991) and CRF stimulates the release of IL-1 and opioids by macrophages (see Karalis et al., 1991). One could speculate that Sertoli cells release testicular IL-1, which in turn causes the release of CRF by Leydig cells and possibly modulates their steroidogenic function. CRF could then stimulate the release of opioids (and/or IL-1) by testicular macrophages and opioids could regulate Sertoli cell function (Gerendai et al., 1986). Macrophages have been shown to be sensitive to androgens and estrogens (Vernon-Roberts, 1969) and thus their secretory function could be influenced by Leydig cell steroids. Ultimately, all these mechanisms are likely aimed at maintaining adequate levels of hormones and nutrients in the certain area of interstitial space in order to optimize sperm maturation.

The other physiological implication of the responsiveness of Leydig cells to IL-1 and macrophage product(s) is that it provides a mechanism by which the immune system can interact with the endocrine system.

It is well accepted that gonadal steroids, including estrogen, progesterone, androgen (Grossman, 1985; Polan, Daniele & Kuo, 1988; Polan et al., 1990), and adrenal steroids (Besedovsky et al., 1986; Buzzetti et al., 1989) can influence the immune and inflammatory responses and the release of IL-1 by macrophages. It would thus be expected that, as in all well regulated physiological processes, IL-1 originating from cells of the immune system can modulate the release of steroids. Whether immune IL-1 is responsible for the changes in gonadal function seen during immune challenge (Christeff et al., 1988; Clemens & Bruot, 1989) remains to be fully demonstrated but several reports point in that direction. Recently, Rivier & Vale (1989) have shown that IL-1 injected peripherally has a direct inhibitory effect on ovarian steroid release. Further support comes from the observation that steroidogenesis by testicular cells from adjuvant-induced arthritic rats is inhibited (Clemens & Bruot, 1989) and that IL-1 production by macrophages from such animals is increased (Johnson et al. 1986). The steroid profiles of male septic shock patients shows an elevation of estrogen levels accompanied by reduced testosterone levels and both adrenal and gonadal steroidogenesis seem to be affected. Interestingly, the pattern of circulating steroids appear to be related

to the chance of survival of the patients (Christeff et al., 1988). During septic shock, TNF (Damas et al., 1989) and IL-1 (Cannon et al., 1990) are elevated and levels have been correlated with the severity of the patient's condition (Cannon et al., 1990). The relationship between the immune and gonadal steroid system may also extend to non-pathological conditions. For instance, the increase in progesterone levels during the luteal phase of the menstrual cycle coincides with an elevation in serum IL-1 (Cannon & Dinarello, 1985) and enhanced ability of monocytes to secrete IL-1 (Polan et al., 1990). Such alteration of circulating IL-1 levels could conceivably influence the immune and inflammatory system although such a link has yet to be examined. It is evident, however, that a better understanding of the interrelation between gonadal steroids and cytokine action and release could prove to have useful clinical implications.

Current knowledge suggests that IL-1 has a dual physiological function: locally produced IL-1 is involved in the paracrine regulation of testicular function and immune IL-1 provides a link between the immune system and gonadal steroidogenesis. Such duality in function for IL-1 has already been infered for other organs such as the thyroid (Ashizawa et al., 1989) and the adrenal cortex (Schultzberg et al., 1989; Winter et al., 1990). This concept will probably extend to other organs since the presence of IL-1 has been identified in numerous 'normal' tissue (Schultzberg et al., 1987).

CONCLUSION

The work presented here demonstrates that IL-1α has a stimulatory effect on basal and submaximally stimulated androgen release and an inhibitory effect on maximally stimulated steroidogenesis in adult rat Leydig cells in primary cultures. These effects are time and concentration dependent and the stimulatory effect is fully reversible. The results indicate that the 17α-hydroxylase/C_{17-20} lyase is the site at which IL-1α exerts its inhibitory effect. No definitive site for the stimulatory effect of IL-1α could be identified but evidence from this work and that of others suggests that IL-1α acts at a step prior to progesterone formation.

This work has shown that IL-1α induces changes in cAMP levels. However, these changes occurs quite some time after IL-1α is added to the culture medium. Furthermore, the changes in cAMP levels are not always accompanied by changes in androgen levels. The data thus suggests that cAMP may not be the primary second messenger involved in the action of IL-1α. This project has provided evidence supporting a role for the lipoxygenase pathway of arachidonic acid metabolism in the stimulatory effect of IL-1α. Blocking the formation of lipoxygenase products results in the gradual disappearance of the stimulatory effect of IL-1α. On the other hand, the formation of lipoxygenase products does not appear to be required for the

inhibitory effect of IL-1α. Therefore, it appears that the stimulatory effect and the inhibitory effect of IL-1α are mediated by separate mechanisms.

This work has provided new information on the effect of IL-1 in Leydig cell steroidogenesis. This is the first time that the stimulatory effect of IL-1 has been clearly demonstrated in purified adult rat Leydig cells. Up to now it had been shown to occur with immature Leydig cell cultures but not with neonatal cells. Results obtained by other researchers with adult cells have been ambiguous. In addition, this is the first report showing an increase in basal cAMP levels in IL-1-treated Leydig cells. Finally, the results using cyclooxygenase and lipoxygenase inhibitors are not only the first evidence for a role of lipoxygenase metabolites in the action of IL-1 in Leydig cells but they are also the first results on the long term effect of inhibition of the lipoxygenase pathway in Leydig cells in culture.

Experiments aimed at determining the effect of testicular macrophages on Leydig cell steroidogenesis provided some interesting findings, albeit preliminary. One such finding was that macrophage culture condition must be carefully monitored if consistent results are to be obtained. In particular, it was found that the preparation of collagenase used for the purification of the macrophages may be an important determinant of the direction of the action of the macrophage conditioned medium on Leydig cells.

In agreement with others, the present results show that testicular macrophages can release a substance(s) capable of modulating androgen release by Leydig cells. The conditioned medium has a stimulatory effect on both basal and LH-stimulated androgen release. This finding is in contrast with the dual effect (stimulation of basal and inhibition of LH-stimulated androgen release) obtained with IL-1α. Consequently, IL-1α may not be the active factor in testicular macrophage conditioned medium.

Taken together, the findings from this study support an involvement of testicular macrophages and of an interleukin-1-like substance in the paracrine regulation of Leydig cell function. Furthermore, the responsiveness of Leydig cells to IL-1α indicate that, in certain conditions, the immune system could modulate gonadal steroidogenesis.

FUTURE DIRECTIONS

Much work remains to be done before we can affirm that we understand the role played by IL-1 in Leydig cell steroidogenesis *in vitro*. First of all, a receptor site for IL-1 has not yet been identified on rat Leydig cells. The existence of specific IL-1 binding sites has been demonstrated on mouse Leydig cells but, ironically, we know very little of the effect of IL-1 on mouse Leydig cells. Identification of the type(s) of IL-1 receptor present on Leydig cells will help determine the mode of action of IL-1 in this system. Now that two types of IL-1 receptors have been cloned, it will not be long before site-directed mutagenesis of the receptor(s) sheds some light on the signal transduction mechanism involved in IL-1 action.

Until such refined tools are available, we could better our knowledge of the signal transduction mechanism of IL-1 in Leydig cell by taking a closer look at the changes in cAMP over time. While the work presented here has shown that cAMP levels remain unchanged in the first 4 h of exposure to IL-1α, these experiments were conducted in the absence of a phosphodiesterase inhibitor. Degradation of cAMP during the 4 h period could have masked an early and/or transient increase in cAMP production.

A long time lag between the onset of IL-1 treatment and a measurable androgen and/or cAMP response was observed and it suggests that the action

of IL-1 requires the synthesis of new proteins. This possibility could be further explored with the use of protein synthesis inhibitors and by the detection of IL-1-induced proteins and/or mRNA.

This project measured the net effect of IL-1 on androgen release. It did not deal with the possibility that this IL-1 could exert is effect on androgen release by altering the pattern of steroids produced by Leydig cells. IL-1 could, for example, reduce the levels of androgen by stimulating their metabolism to estrogen. Or it could stimulate metabolism of androgen precursors into steroids that cannot be metabolized into androgens. Studying the profile of steroids produced by IL-1-treated Leydig cells may reveal enhanced activity of certain steroid enzymes. Such information could shed some insight on the possible impact of the action of IL-1 in the *in vivo* setting.

The role of arachidonic acid and its metabolites in Leydig cell steroidogenesis has been the subject of some controversy over the years. The results obtained in this project indicate that inhibiting the lipoxygenase pathway of arachidonic acid metabolism with NDGA interferes with 'normal' *in vitro* steroidogenesis. Chemical inhibitors are not as specific as one might hope for, and thus additional experiments using other lipoxygenase inhibitors would strengthen the conclusion drawn from the work with NDGA. Further, information could be gained by adding exogenous lipoxygenase metabolites to

cultures treated with lipoxygenase inhibitors or by measuring the production of arachidonic acid metabolites in IL-1-treated Leydig cells.

Despite the variability in the effect of the testicular macrophage conditioned medium on Leydig cell steroidogenesis, there is no doubt that testicular macrophages have the ability to release a substance or substances that can modulate Leydig cell function. Some of the known macrophage products have already been shown to modulate Leydig cell steroidogenesis and thus are 'prime candidates' as the active agent(s) in the TMCM. Whether they are present in the TMCM has not yet been evaluated. For example, TMCM could be assayed for IL-1 or TNF or, antibody to these molecules could be added to the TMCM. Similar approaches could be used to verify the presence of other known macrophage products. Measurements of mRNA for these molecules could also be used. Because the procedure for the isolation of macrophages likely results in their activation and thus in the production of substances that they may not release *in vivo* it will be important to verify that the active agent(s) in the TMCM is also produced *in vivo*. *In situ* hybridyzation, for example, could be used to detect the presence of mRNA for such agent(s) in non-activated macrophages. An other interesting line of work would be to investigate whether Leydig cell products such as steroids, CRF or opioids for example, can modulate the activity of the TMCM and the release of active substance(s) by macrophages. Refinement of the coculture technique will be

necessary before further work on the importance and impact of cell-cell contact betweem Leydig cells and macrophages can take place.

Macrophages and IL-1 have long been considered as playing a role only in the immune system. In recent years, research has provided much evidence showing that macrophages and IL-1 may also play a key role in the paracrine regulation of normal physiological functions. A close link between the endocrine system and the immune system appears to exist and macrophages and IL-1 could be key players in the communication between the two systems. The work presented in this thesis shows that both IL-1 and testicular macrophages can modulate Leydig cell function. Future work should clarify the mode of action of IL-1 and identify the macrophage factors responsible influencing androgen release as well as define the mechanisms that regulate their release. Finally, to make the picture complete, all these findings will have to be looked at in the context of the *in vivo* regulation of Leydig cell and testicular function.

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