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THE ROLE OF SECOND MESSENGER INTERACTIONS IN THE CONTROL OF ANDROGEN PRODUCTION IN THE MOUSE LEYDIG CELL

by

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Submitted in Partial Fulfillment of the Requirements
for the degree of Doctor of Philosophy

at

Department of Physiology and Biophysics
Dalhousie University
Halifax, Nova Scotia
February, 1991

ISBN 0-315-71485-7

DEDICATION

I dedicate this thesis is to my wife, Susan, and to my father, Richard James Hipkin.

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ABSTRACT

The goal of the studies in this thesis was to characterize the role of second messenger interactions in regulation of androgen (T) output by the Leydig cell (LC).

Analogues of cyclic adenosine 3',5'-monophosphate (cAMP) selective for either binding site (S1 and S2) on the regulatory subunits of cAMP-dependent protein kinases (PK-A) were used to assess the role of type 1 (T1) and type 2 (T2) PK-A in LC function. As S1 and S2 exhibit positive cooperativity, coexposure to analogue pairs will synergistically increase T output should T1 or T2 PK-A be present. Both T1 and T2 PK-A were active in the LC, though T1 PK-A activity was predominant. Coincubation with cAMP, luteinizing hormone (LH) or forskolin also synergistically increased T though the response with forskolin was reduced suggesting cAMP-independent activities.

Although atrial natriuretic factor (ANF) stimulates T output via cyclic guanosine 3',5'-monophosphate (cGMP), coexposure of LC to ANF and LH or ANF/cGMP and the same cAMP analogues resulted in a synergistic increase in T production suggesting cyclic nucleotide interaction mediates a cooperative hormonal control of the mouse LC in vivo.

The role of calmodulin (CaM) in LC function was assessed using a CaM antagonist, N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W7) and a multichambered cell perifusion system. Exposure to W7 caused an initial nonspecific inhibition followed by a CaM-dependent increase in T synthesis suggesting the presence of a novel inhibitory CaM-sensitive process in the LC. The response did not involve inhibition of a CaM-dependent phosphodiesterase (PDE) but could be abolished with a phospholipase A_2 inhibitor and mimicked by a lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). The response to W7 and NDGA co-infusion was not additive, suggesting a common mechanism of action, possibly mediated by arachidonic acid.

LIST OF ABBREVIATIONS

α_i	Inhibitory α-subunit
α_s	Stimulatory α-subunit
AA	Arachidonic acid
AC	Adenylate cyclase
AHA	Aminohexylamino-cAMP
ANF	Atrial natriuretic factor
ANOVA	Analysis of variance
ASA	Acetylsalicilic acid
ATP	Adenosine triphosphate
B	N^6-Benzoyl-cAMP
BSA	Bovine serum albumin
CaM	Calmodulin
cAMP	Cyclic adenosine 3',5'-monophosphate
cGMP	Cyclic guanosine 3',5'-monophosphate
CPM	Counts per minute
DDF	1,9-Dideoxyforskolin
DG	1,2-Diacylglycerol
EC_{50}	Effective drug concentration to elicit a half-maximal response
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
G_i	Inhibitory G-protein
G_s	Stimulatory G-protein
G-protein	GTP-regulated proteins
GC	Guanylate cyclase

GDP	Guanosine diphosphate
GnRH	Gonadotropin releasing hormone
GH	Growth hormone
GTP	Guanosine triphosphate
hCG	Human chorionic gonadotropin
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
3β-HSD	3β-Hydroxysteroid dehydrogenase
IC_{50}	Drug concentration which is effective in inhibiting a response by 50%
I.D.	Inner diameter
IGF-I	Insulin-like growth factor I
IgG	Immunoglobulin G
IL-1α/β	Interleukin 1α/β
IP_3	Inositol 1,4,5-triphosphate
LH	Luteinizing hormone
MIX	3-Isobutyl-1-methylxanthine
M_r	Relative molecular weight
NDGA	Nordihydroguaiaretic acid
NSB	Non-specific binding
O.D.	Outer diameter
25-OH-chol	25-Hydroxycholesterol
$P450_{scc}$	Cholesterol side-chain cleavage cytochrome P450 enzyme
$P450_{17\alpha}$	Cytochrome $P450_{17\alpha}$
PDE	Phosphodiesterase
PG	Prostaglandins
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PK-A	3',5'-cyclic adenosine monophosphate-dependent protein kinase
PK-C	Calcium/phospholipid-dependent protein kinase
PK-G	3',5'-cyclic guanosine monophosphate-dependent protein kinase
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PP	Protein phosphatases
R^1	Type 1 cAMP-dependent protein kinase regulatory subunit
R^2	Type 2 cAMP-dependent protein kinase regulatory subunit
R_i	Inhibitory receptor
R_s	Stimulatory receptor
RIA	Radioimmunoassay
S_1	Site 1 cAMP-binding site
S_2	Site 2 cAMP-binding site
SCP_2	Sterol carrier protein$_2$
SER	Smooth endoplasmic reticulum
SR	Synergism ratio
T1	Type 1 cAMP-dependent protein kinase
T2	Type 2 cAMP-dependent protein kinase
TC-199	Tissue culture medium-199
TFP	Trifluoperazine
TGF-β	Transforming growth factor-β
W5	N-(6-aminohexyl)-1-napthalenesulfonamide
W7	N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. W.H. Moger, for his help and support during my Ph.D. training. In addition, I would like to offer my thanks to my supervisory committee for their help and advice. I would also like to acknowledge the technical and moral support of Alice Giles, Marc Boylan, Mereille Limoges and Dr. Michael McDonald.

I am very grateful to all my cohorts and fellow graduate students for their friendship, tolerance and general debauchery. Special thanks to my family for their love and support over the last 30 years. To my wife, Sue, whom I love and respect.

I. INTRODUCTION

Testicular production of the steroid, testosterone, is vital for normal sexual development and function in the male. This potent androgen acts both as a paracrine factor in supporting spermatogenesis in the adjacent seminiferous tubules (Steinberger and Steinberger, 1974) and as an endocrine hormone to initiate and support secondary sexual characteristics (Wilson et al., 1981). The Leydig cell, located in the testicular interstitium, is the primary source of testosterone in the male (Christensen and Mason, 1965). Comprising approximately 1-5% of the testicular volume in the rodent and 15% in the primate (Fawcett et al., 1973), the Leydig cell can be distinguished from other testicular cell types by its round, acentric nucleus and by the abundance of smooth endoplasmic reticulum (SER) (Christensen, 1975). The presence of large amounts of SER is functionally significant as it, along with the mitochondria encompass the enzymatic apparatus responsible for steroid synthesis (steroidogenesis). Indeed, the steroidogenic activity of the Leydig cell has been shown to directly correlate with the amount of SER present in the cell cytoplasm (Ewing and Zirkin, 1983).

The production of testosterone in the testes is under the tight control of a variety of endocrine and paracrine hormones or "first messengers". In most cases, these

hormones are proteins and as such, cannot easily pass through the lipid bilayer of the cell membrane. Due to this constraint, external control of cell function usually occurs indirectly via various intracellular regulators or "second messengers" generated upon binding of the hormone to a specific membrane receptor (Catt and Dufau, 1976).

Many of the attempts to isolate and study these intracellular messenger cascades fail to account for potential interaction between second messenger systems. The tendency to categorize these messenger systems as simple linear pathways probably more accurately reflects on the difficulties inherent in deciphering their complexities than on the true nature of messenger organization. The goal of this project is to investigate the potential for messenger "crosstalk" within the mouse Leydig cell and to determine the relevance of this phenomenon in the control of testicular androgen production.

A. Steroidogenesis in the Leydig cell

Steroidogenic enzymes catalyze the conversion of the sterol, cholesterol to sex steroids in the testis (Payne, 1990) and the ovary (Fortune and Armstrong, 1978) and to corticosteroids in the adrenal cortex (Hall, 1985). Although they are structurally and functionally dissimilar, the steroids produced by these tissues are synthesised by

enzymatic processes which share certain common characteristics. (Gower and Cooke, 1983). In all steroidogenic tissues, cholesterol undergoes a series of oxidation and cleavage processes which sequentially convert the 27 carbon (C) sterol to 21C progestins. In the ovary, the thecal cells can utilize progestins to synthesize androgens which diffuse to the granulosa cells for conversion to phenolic 18C estrogens (Fortune and Armstrong, 1978). The adrenal cortex has the capability to synthesize a whole host of different biologically active steroids, including 21C glucocorticoids from the fasciculata cells and mineralocorticoids from the glomerulosa cells (Hall, 1985). In the testis, androgens are usually the primary steroid product although large quantities of estrogens are also produced in some species (Raeside and Renaud, 1983). Steroidogenesis in the Leydig cell can occur via two parallel processes, referred to as the 4-en-3-oxo ($\Delta 4$) and the 5-ene-3β-hydroxy ($\Delta 5$) pathways (Gower and Cooke, 1983). As the $\Delta 4$ pathway predominates in the rodent (Shikata et al., 1964; de la Torre et al., 1977), discussion will be limited to that pathway.

The Leydig cell derives cholesterol from three different sources: de novo synthesis from acetate, cytoplasmic lipid droplets containing cholesterol esters and exogenous cholesterol associated with serum lipoproteins (Quinn et al., 1981). Transported to the mitochondria by

sterol carrier protein$_2$ (Chanderbhan et al., 1982), cholesterol then acts as a substrate for the cholesterol side-chain cleavage cytochrome P450 enzyme ($P450_{SCC}$) located in the inner mitochondrial membranes (Payne, 1990). The $P450_{SCC}$ enzyme catalyses the cleavage of the C20-C22 bond of cholesterol, yielding the 21C steroid, pregnenolone (Hall, 1966). Pregnenolone is then rapidly converted to progesterone by a 3β-hydroxysteroid dehydrogenase/isomerase enzyme (3β-HSD) located in the SER (Fan and Troen, 1975). Within the SER, progesterone acts as substrate for another cytochrome enzyme system, cytochrome $P450_{17\alpha}$ ($P450_{17\alpha}$), which catalyses two reactions, acting as both a 17α-hydroxylase and as a C_{17-20} lyase to cleave the C17-C20 bond, converting the 21C progesterone to 19C androgens (Fevold et al., 1989). The 17C keto- moiety on the resulting steroid molecule is subsequently reduced by 17-ketosteroid reductase, converting the weak androgen, androstenedione to the biologically active, testosterone (Bogovich and Payne, 1980).

B. Hormonal Control of Leydig cell function

A large number of factors have been shown experimentally to influence the production of androgens by the Leydig cell. Some of these factors act as acute activators of steroidogenesis while others act as permissive

agents which modulate the response to those steroidogenic activators. The more acutely stimulatory factors include luteinizing hormone (LH; Catt et al., 1972), atrial natriuretic factor (ANF; Pandey et al., 1985; Bex and Corbin, 1985; Foresta et al., 1987), epinephrine (Moger and Murphy, 1982; Anakwe and Moger, 1984) and gonadotropin releasing hormone (GnRH; Sharpe and Cooper, 1982) though only the influence of LH has as of yet been conclusively shown to be of physiological relevance. Many of the more subtle modulators of androgen production are growth factors such as epidermal growth factor (EGF; Ascoli, 1981), insulin and insulin-like growth factor I (Lin et al., 1986; Moger and Murphy, 1987), transforming growth factor-β (TGF-β; Lin et al., 1987) and fibroblast growth factor (Sordoillet et al., 1988). Some of these mediators and others such as interleukin 1α/β (Lin et al., 1988, Calkins et al., 1990) and arginine vasopressin (AVP; Adashi and Hseuh, 1981) are probably acting as intratesticular regulators of Leydig cell function rather than in the classic endocrine manner.

1. Luteinizing hormone

Luteinizing hormone (LH) is widely accepted as the predominant stimulatory mediator of mammalian Leydig cell steroidogenic function (Catt et al., 1972; Mendelson et al., 1975; Keeney et al., 1988). The synthesis and secretion of

LH from the anterior pituitary is controlled by the pulsatile release of the hypothalamic decapeptide, GnRH (Aiyer et al., 1973). The release of GnRH and LH is in turn influenced by negative feedback of sex steroids and peptides from the testis (Gay and Dever, 1971). A pulsatile rather than continuous release of LH into the bloodstream is vital for the control of androgen production as chronic exposure to high levels of gonadotropins has beem shown to elicit the receptor down regulation on the Leydig cell and the loss of gonadotropin responsiveness (Ascoli, 1981; Rebois and Fishman, 1984).

Acting via second messengers, LH stimulates testosterone production by the mammalian Leydig cell, both acutely and chronically (Catt et al., 1972; Mendelson et al., 1975; Keeney et al., 1988). This stimulatory influence is mediated at a number of different levels within the cell. The gonadotropin has been shown to maintain the volume of SER and cytoplasm within the Leydig cell (Wing et al., 1984; Keeney et al., 1988) while enhancing the delivery of cholesterol to the $P450_{scc}$ enzyme in the mitochondria (Hall et al., 1981), the rate-limiting step in steroidogenesis (Privalle et al., 1983). The synthesis of $P450_{scc}$ enzyme was also found to be LH-sensitive (Mason et al., 1984) as was the maintenance of the enzymatic apparatus responsible for the conversion of progesterone to the 19C androgens (Wing et al., 1984). The expression of $P450_{17\alpha}$ activity

disappeared in the absence of the gonadotropin in both the mouse and rat Leydig cell (Malaska and Payne, 1984; Wing et al., 198·).

During pregnancy in some species (human, horse), the structural and functional integrity of the cyclic corpus luteum is prolonged by an LH-like chorionic gonadotropin synthesized within the placental membranes (Jaffe, 1978). Thought to bind at the LH receptor (Dufau et al., 1977), human chorionic gonadotropin (hCG) has been used extensively as an LH analogue for in vitro studies on the influence of gonadotropins on Leydig cell function (Mendelson et al., 1975; Dufau et al., 1977; Ascoli, 1981; Rebois and Fishman, 1984; Pereira et al., 1987; Inoue and Rebois, 1989).

2. Atrial natriuretic factor

ANF was originally isolated from rat atria and characterized as a group of related polypeptide hormones with both diuretic and natriuretic activities (deBold et al., 1981). ANF was shown to manifest these activities, in part, by inhibiting aldosterone secretion by adrenal glomerulosa cells (Kudo and Baird, 1984). Continued characterization of its biological functions revealed that ANF also influenced the function of a number of other steroidogenic tissues (Racz et al , 1985) including both tumour-derived and normal Leydig cells (Pandey et al., 1985;

Bex and Corbin, 1985; Pandey et al., 1986b; Foresta et al., 1987). Nanomolar concentrations of ANF inhibited gonadotropin-stimulated progesterone synthesis in murine Leydig tumour cells (Pandey et al., 1985). Although an analogous response was reported in one study using freshly isolated mouse Leydig cells (Pandey et al., 1986), a similar study by Mukhopadhyay and coworkers (1986a) did not support these findings. However, both studies (Pandey et al., 1986b; Mukhopadhyay et al., 1986a) supported earlier findings (Bex and Corbin, 1985) that ANF ($\geq$ 1 nM) stimulated freshly isolated mouse Leydig cells, but did so to an extent rivaling that noted upon exposure to LH (Pandey et al., 1986a). Subsequent studies with the rat Leydig cell revealed that much lower concentrations of ANF (10^{-11} M) increased basal androgen production although this effect was lost with higher concentrations of the peptide (10^{-9} M; Foresta et al., 1987). This effect was mediated via specific, saturable ANF receptors which have been located on the plasma membrane of both rat and mouse Leydig cells (Foresta et al., 1987; Pandey et al., 1986a). While the stimulatory effect of ANF in the rat occurred at concentrations which may or may not arise in its role as a blood-borne hormone (Anderson and Bloom, 1986; Thibault et al., 1988), it would seem more likely that ANF could be acting as an intratesticular paracrine factor both in the mouse and the rat. This possibility was supported by a study

in which both an ANF prohormone (ANF 2-126) and its messenger ribonucleic acid (mRNA) sequence were isolated from the rat testis (Vollmar et al., 1990). No similar study has been attempted in the mouse. All of these findings suggest that ANF may well be an important, albeit little studied, factor in the control of testicular androgen production.

3. Arginine-vasopressin

Adashi and Hseuh (1981) provided the original evidence that AVP and pressor-selective analogues inhibited gonadotropin-stimulated androgen production by rat interstitial cells. While this reduction in Leydig cell responsiveness involved reduced generation of second messengers in response to gonadotropin/receptor interaction (Adashi et al., 1984), an AVP-mediated inhibition of C_{17-20} lyase activity was also demonstrated (Adashi et al., 1982). The stimulation of second messenger cascades by AVP has not been fully characterized in the Leydig cell although there is evidence in Leydig tumour cells that a rise in intracellular Ca^{2+} is involved (Ascoli et al., 1989).

4. Interleukin-1α/β

The immunological mediator, interleukin 1α/β (IL-1α/β) has been shown to inhibit the responsiveness of rat Leydig cells to stimulation by gonadotropins (Calkins et al., 1988; Calkins et al., 1990) at a point beyond the generation of second messengers. Preliminary findings by Moore and Moger (in press) suggested that in addition to the inhibition of LH responsiveness, IL-1α acts to support basal androgen production by cultured Leydig cells, a cell type notorious for its loss of normal steroidogenic function during prolonged culture (Mather, 1980; Murphy and Moger, 1982). The presence of large numbers of macrophages in the testicular interstitium (approximately 20% of interstitial cells; Yee and Hutson, 1983) suggests that interleukins may well act as a paracrine factor in intratesticular cell-cell communication.

5. Growth factors

A number of growth factors have been shown to support Leydig cell steroidogenic function. Insulin-like growth factor I (IGF-1), insulin, growth hormone (GH) and fibroblast growth factor have all been shown to potentiate the steroidogenic response of cultured rat Leydig cells to gonadotropins (Lin et al., 1986; Moger and Murphy, 1987;

Sordoillet et al., 1988; Horikawa et al., 1989). As neither hGH nor insulin augmented the maximal IGF-I-mediated enhancement of gonadotropin responsiveness, it would seem that these growth factors act through the same intracellular mechanisms.

Transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) do not influence Leydig cell function in the same manner as do the other growth factors. TGF-β was found to attenuate rather than potentiate the steroidogenic response to hCG and 8-bromo-cAMP (Lin et al., 1987) while EGF had a triphasic influence on rat Leydig cell function. Within the initial 5 minutes of exposure, EGF inhibited hCG-stimulated androgen production in MA-10 tumour Leydig cells while intermediate exposure time to EGF (1-8 hours) stimulated steroidogenic function. Prolonged exposure (8-48 hours) instigated LH/hCG receptor downregulation (Ascoli and Segaloff, 1989). This unusual time-dependent relationship provides an illustration of the complexity inherent to the regulation of cellular activities by hormonal factors.

A number of these growth factors can directly influence cell function via membrane-bound tyrosine kinases. EGF, IGF-I and insulin all interact with transmembane receptor proteins encompassing an inducible protein kinase which exclusively phosphorylates tyrosine residues of target proteins (Sefton and Hunter, 1984).

C. The second messengers

A variety of intracellular factors have been implicated as putative second messengers in the hormonal control of steroidogenesis in the Leydig cell. These factors can be loosely categorized into three different messenger systems: the cyclic nucleotides, ionic calcium and associated binding proteins and the eicosanoids. This convenient categorization is, as is usually the case, simplistic in that it implies a high degree of compartmentalization. To overlook or minimalize the potential importance of interaction or "crosstalk" between the messenger systems is unwise. One goal of this literature review is to highlight the documented incidences of messenger crosstalk and to address how this interaction could effect androgen production in the mouse Leydig cell. For a more extensive literature review on second messenger interactions, refer to Role and Schwartz (1989).

1. The cyclic nucleotides

The cyclic nucleotide second messengers, cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) are derived by hydrolytic cleavage of phosphate bonds in adenosine triphosphate (ATP) and guanosine triphosphate (GTP), respectively. Both of

these second messengers can alter cellular metabolism through the activation of specific cAMP- or cGMP-dependent phosphotransferases which, through phosphorylation, increase or decrease the activity of target proteins (usually enzymes). Unlike the growth factor receptors which phosphorylate tyrosine residues (Yarden and Ullrich, 1988), the cyclic nucleotide-dependent protein kinases have been shown to phosphorylate serine or threonine residues (Krebs, 1986). In either case, the activities of these substrate protein effectors were directly dependent upon their state of phosphorylation. As cAMP-mediated processes have been more exhaustively studied, discussion shall begin with this particular cyclic nucleotide messenger.

1a) cAMP

Exposure to LH, hCG, or epinephrine stimulated the synthesis of cAMP in the Leydig cell, (Catt et al., 1972; Mendelson et al., 1975; Dufau et al., 1978; Moger et al., 1982) thereby triggering a variety of intracellular events in the Leydig cell, including the activation of the P450 steroidogenic enzymes (Malaska and Payne, 1984; Anakwe and Payne, 1987). Certain other hormonal factors, such as AVP, ANF and EGF can reduce androgen production through the inhibition of cAMP synthesis (Adashi et al., 1984; Pandey et al., 1985; Ascoli and Segaloff, 1989).

The mechanism by which hormone/receptor interaction stimulates cAMP synthesis is quite complex and has been extensively studied (see Levitzky, 1987 and Fig. 1 for review). Binding of stimulatory hormonal factors to their specific membrane-bound receptors has been shown to initiate a conformational change in the receptor structure which facilitates its interaction with adenylate cyclase (AC), the enzyme responsible for cAMP synthesis. Interaction of these stimulatory receptors (R_s) with AC has been shown to activate the catalytic domain of the enzyme and subsequently, the generati of cAMP (Levitzky, 1987). If the adenylate cyclase should interact with an inhibitory receptor (R_i), the activity of the AC enzyme would be reduced rather than enhanced.

Interaction between R_s/R_i and AC is indirect, employing GTP-regulated proteins (G-proteins) embedded in the lipid bilayer as transducer elements. It should be noted that these G-proteins have been found to act as transducers for receptor-mediated events which utilize second messengers other than the cyclic nucleotides ie. the eicosanoid cascade (Axelrod et al., 1988) and ionic calcium (Snyderman et al., 1986). G-proteins were found to exist as heterotrimers comprised of a hydrophobic $\beta\gamma$ dimer reversibly bound to a hydrophilic α-subunit with an associated guanosine diphosphate (GDP) moiety (see Spiegel, 1987, for review). Hormones such as LH which activate Leydig cell AC

activity do so via stimulatory G-proteins (G_S) (Dix et al., 1982) closely associated with the hydrophobic catalytic unit of the transmembrane adenylate cyclase protein (Arad et al., 1984). Binding of the ligand to R_S facilitates a GTP-for-GDP exchange on the stimulatory α-subunit (α_S) and the subsequent dissociation of the G_S trimer into the $\beta\gamma$- and the α_S-GTP components, the latter remaining associated with the catalytic subunit of AC (Arad et al., 1984). The activated α_S-subunit could then induce a conformational change in the AC catalytic domain facilitating the conversion of intracellular ATP to cAMP. The interaction of G_S and AC can be also elicited artificially with cholera toxin which ADP-ribosylates the $G_S\alpha$ subunit, stabilizing the GTP-bound form, resulting in chronic adenylate cyclase activation (Ross and Gilman, 1980). Experimental use of cholera toxin is valuable in assessing the role of G_S proteins in hormonal control of cellular functions.

The G-proteins associated with inhibitory receptors are virtually identical to G_S except that the G_i proteins contain an inhibitory α_i protein that does not form a complex with the catalytic unit of the enzyme (Smigel, 1986). Instead, GTP/GDP exchange on the α_i entity and subsequent trimer disassociation acts only to increase the availability of free $\beta\gamma$ subunits, increasing the likelihood of reassociation with any available α_S subunits thereby decreasing their influence on catalytic activity (Levitzky,

1987). In this manner, the inhibitory receptor species inhibit AC activity indirectly by attenuating the stimulatory influence of G_s. Therefore, reduction of G_i influence on AC activity could provide a mechanism for the indirect stimulation of cAMP generation. Pertussis toxin was found to stimulate cAMP synthesis in this manner as it ADP-ribosylated the $G_i\alpha$ subunit which stabilizes the protein in its associated, GDP bound state thereby preventing the inhibition of AC activity (Gilman, 1984). As a result, pertussis toxin can be utilized experimentally to assess the role of G_i in the hormonal control of cell function (Adashi et al., 1984; Platts et al., 1988).

Findings by Adashi and coworkers (1984) provided the first evidence that a tonically inhibitory G protein influenced steroidogenic function in the Leydig cell. They demonstrated that pertussis toxin enhanced the steroidogenic response of rat interstitial cells to hCG while completely abolishing the inhibitory effects of AVP (Adashi et al., 1984). It cannot be said with certainty that this effect was not mediated by some cell type other than Leydig cells as an unpurified testicular cell preparation was utilized. Platts and coworkers (1988) established the presence of an inhibitory G protein impinging on adenylate cyclase activity in the purified rat Leydig cells. They showed that exposure to pertussis toxin for 24 hours potentiated both basal and LH-stimulated cAMP production and ADP-ribosylated

proteins within the Leydig cell membrane.

The α subunits also have hormone-dependent GTPase activity (Cassel and Selinger, 1976) which hydrolyses the GTP to GDP, deactivating the α subunit which reassociates with the $\beta\gamma$ subunit. In doing so, the ligand/receptor/G-protein complex has intrinsic self-limiting capabilities which act to control the response, be it inhibitory or stimulatory.

The generation of cAMP is only the first step in the messenger cascade, however. Each R_S/AC complex will result in the generation of a large number of cAMP molecules which will, in turn, activate an intracellular phosphotransferase enzyme, cAMP-dependent protein kinase (PK-A).

The two predominant isoenzymes of PK-A, referred to as type 1 (T1) and type 2 (T2) PK-A, differ in certain functional and structural characteristics. Both the T1 and T2 isoenzymes are heterotetramers comprised of two regulatory subunits (M_r=49,000-55,000) joined at the amino terminus by disulphide bonds (Bubis et al., 1987) and each associated with a catalytic subunit (M_r≈40,000) (Nairn et al., 1985). Binding of cAMP to the regulatory domains has been shown to release and, therefore, activate the catalytic domain, which subsequently phosphorylate target proteins (Hofmann, 1980). While the two isoenzymes appear to utilize common catalytic subunits (Hanks et al., 1988), the regulatory subunits are type-specific (R^1 and R^2) and confer

different functional characteristics on to the kinases. For example, the R^2 subunit ($M_r \approx 52,000-55,000$) was shown to be very susceptible to autophosphorylation (Rosen and Erlichman, 1975). Autophosphorylation is an intramolecular process with important functional consequences as it impairs the ability of the regulatory subunit to recombine with the catalytic subunit resulting in enhanced phosphotransferase activity (Rosen et al., 1977). Self-phosphorylation may, in part, explain the increased sensitivity of the T2 phosphotransferase to cAMP activation (K_A = 1.8 nM) relative to the T1 isoenzyme (K_A = 18 nM; Vardanis, 1980) as the T2 isoform exists primarily in the phosphorylated state (Rangel-Aldao et al., 1979).

The R^1 subunit ($M_r \approx 49,000$), while somewhat less susceptible to phosphorylation (Taylor, 1989) has a high affinity allosteric binding site for MgATP (Hofmann et al., 1975). A number of distinct gene products also exist within each class such as $R^1\alpha$, common in most tissues (Lee et al., 1983) and the more tissue-specific $R^2\beta$ (Jahnsen et al., 1986).

The regulatory subunits do share some common characteristics, however. For example, a protolytically sensitive "hinge" region exists in the amino-terminal region of each regulatory subunit. This sequence was found to contain both a substrate-like sequence, which was the site of interaction between the regulatory protein and the

catalytic subunit and the phosphorylation sites (Taylor, 1989). Two tandem cAMP binding sites (S1 and S2) were shown to be highly conserved on all regulatory subunits though the binding affinity of each site for cAMP was type-dependent (Corbin and Lincoln, 1978).

Development of cAMP analogues selective for each of the two binding sites on the R^1 and R^2 domains has provided an opportunity to assess the role of each PK-A isoenzyme in various metabolic processes (Beebe et al., 1988). The functional assessment of the cAMP-dependent protein kinases with these analogues was possible because the two cAMP binding sites have been shown to exhibit positive cooperativity (Rannels and Corbin, 1981), in that binding at one site increased binding at the other site. Therefore, the functional involvement of a PK-A isoenzyme within a particular cellular activity would be evident should exposure to cAMP analogues selective for S1 and S2 of that isoenzyme elicit a synergistic rather than additive response (Corbin et al., 1988). This type of assessment has not been attempted in Leydig cells although both PK-A isoenzymes have been physically isolated from rat Leydig cells and murine Leydig tumour cells (Cooke et al., 1976; Peirera et al., 1987). It was determined that the type 2 PK-A was responsible for only a minor percentage of the total detectable phosphotransferase activity (15-20%) in the Leydig tumour cells (Peirera et al , 1987). To reiterate,

however, the functional importance of either PK-A isoenzyme in Leydig cell steroidogenesis has yet to be determined.

Although it has been well documented that LH stimulates steroidogenic activity in the Leydig cell through the cAMP second messenger system (Mendelson et al., 1975; Cooke et al., 1976), gonadotropin-stimulated androgen production has been detected prior to any discernable elevation in the levels of the cyclic nucleotide both in freshly isolated (Mendelson et al., 1975; Cooke et al., 1976) and tumour Leydig cells (Peirera et al., 1987). However, use of a phosphorothioate analogue of cAMP, R_p-cAMP, as a competitive cAMP antagonist (Eckstein, 1985) inhibited the steroidogenic response to these low levels of hCG, suggesting that the cAMP cascade was involved (Peirera et al., 1987). The inability to discern any significant changes in intracellular cAMP levels paralleling the gonadotropin stimulation of androgen synthesis could provide a clue to compartmentalization of the cAMP-dependent protein kinases. It is possible that cAMP generation in response to low stimulatory levels of gonadotropins could only be sufficient to activate PK-A in the immediate vicinity of the site of synthesis (ie. the cytoplasmic side of the plasma membrane) but not sufficient to change the measurable levels of cAMP within the whole cell. This would be analogous to setting off a fire alarm by placing a small flame within close proximity to the heat detector but without the flame

having any significant effect on the temperature within the room.

Cellular activation by cAMP and its associated kinases can be reversed through a number of different processes. Reduction of cellular concentrations of cAMP is achieved by enzymatic degradation of the cyclic nucleotide by phosphodiesterase (PDE) enzymes (Weishaar, 1987). In the classic model, a single cyclic nucleotide PDE converted the active cAMP messenger to the inactive AMP form which was then converted back to ATP by mitochondria (Weishaar, 1987). It soon became apparent, however, that the classic model was inadequate and that there was actually several PDE isoforms with different functional characteristics (Thompson and Appleman, 1971; Weishaar, 1987). Indeed, three major PDE isoenzymes have been isolated and characterized on the basis of their substrate specificity and their sensitivity to Ca^{2+}/calmodulin and cGMP. Type I and II PDE were found to hydrolyse cAMP and cGMP to the same degree while the type III PDE selectively degraded cAMP (Weishaar et al., 1985). Type I PDE has been referred to as the "calmodulin-stimulated phosphodiesterase" as its activity was shown to more than double in the presence of Ca^{2+}/calmodulin (Weishaar et al., 1985). The activity of type II and III PDE enzymes was influenced by cGMP such that type II activity was stimulated somewhat while cAMP hydrolysis catalyzed by type III PDE was drastically reduced (Weishaar, 1987).

Subsequent research demonstrated that the PK-A catalytic units phosphorylated PDE and increased the rate of cAMP catabolism (Gettys et al., 1987). This would provide a negative feedback apparatus by which the cAMP cascade would be self-limiting (Corbin et al., 1988).

A fairly large amount of cAMP may also be removed from the cellular environment through active, saturable export to the extracellular space (Brunton and Heasley, 1988). Data from one of the original comprehensive studies on the role of cAMP in Leydig cell steroidogenesis was suggestive of active cAMP export (Dufau et al., 1977). Experiments in which rat interstitial cells were exposed to highly stimulatory levels of hCG revealed that the rate of cAMP accumulation in the extracellular compartment was an order of magnitude greater than that within the cell. As the cells were incubated in the presence of a PDE inhibitor, enhanced intracellular cAMP degradation was not a factor. Even accounting for the saturation of available intracellular binding sites, the rate of increase in intracellular and extracellular cAMP should be more or less parallel There has been no attempt, as of yet, to investigate the role of cAMP export in the control of androgen production in the Leydig cell.

The state of substrate phosphorylation is actually directly dependent on the relative activities of two phosphotransferases: the protein kinases and the

phosphoprotein phosphatases (PP) which actively dephosphorylate kinase substrates. The PP enzymes were grouped into two classes, type 1 (PP-1) and type 2 protein phosphatases (PP-2A, -2B and -2C) (Nairn et al., 1985). The activity of PP-1 is regulated by PK-A which activated protein phosphatase inhibitors thereby amplifying cAMP-mediated events as well as providing a mechanism through which cAMP could modulate the phosphorylation state of other protein kinase substrates (Cohen, 1982). The PP-2 enzymes were found to require Ca^{2+} and, in some cases, calmodulin and were insensitive to the protein phosphatase inhibitors which modulated PP-1 activity (Nairn et al., 1985). PP-2B, referred to as calcineurin (Wang and Desai, 1977) or calmodulin-dependent protein phosphatase (Tung, 1986), was shown to consist of two subunits, A and B (Klee and Krinks, 1978). Subunit A interacted with Ca2+/CaM (Klee and Krinks, 1978) while subunit B was a calcium-binding protein somewhat homologous to CaM (Aitken et al., 1984). All of the protein phosphatases exhibit fairly wide substrate specificities. It was established that PP-2B dephosphorylated phosphoseryl/threonyl-containing proteins such as the regulatory subunit of type 2 PK-A (Blumenthal and Krebs, 1983) as well as phosphotyrosine-containing proteins (Chernoff et al., 1984). The protein phosphatases themselves can also act as phosphotransferase substrates with PP-2B being phosphorylated by protein kinase C though

Fig. 1. The cyclic nucleotide second messenger cascades. This figure schematically illustrates the mechanisms through which stimulatory and inhibitory receptors and associated G proteins (R_s/G^s and R_i/G_i) mediate the hormonal control of cellular function. Both cAMP synthesis by adenylate cyclase (AC) and the ANF R_s/guanylate cyclase (GC) complex are portrayed. Activation of cyclic nucleotide-dependent protein kinases and subsequent substrate phosphorylation are also represented. Note the mechanisms opposing cellular activation which include cyclic nucleotide export or hydrolysis by phosphodiesterase (PDE) enzymes as well as substrate dephosphorylation by protein phosphatases. (+) or (-) represents stimulation and inhibition, respectively while (?) suggests putative involvement.

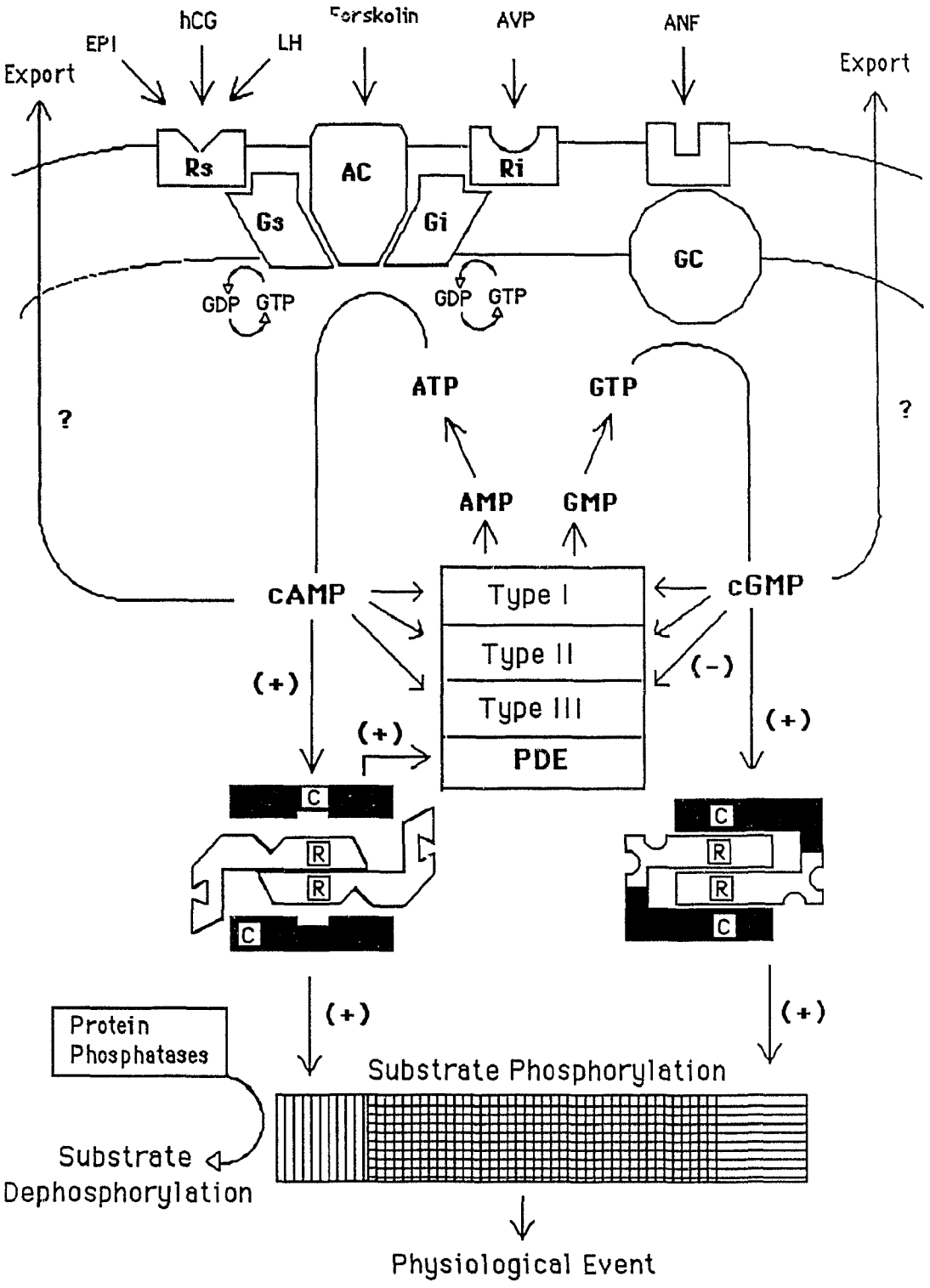

EPI
hCG
LH
Forskolin
AVP
ANF
Export
Export
Rs
AC
Ri
Gs
Gi
GC
GDP GTP
GDP GTP
?
?
ATP
GTP
AMP
GMP
cAMP
cGMP
Type I
Type II
Type III
PDE
(+)
(+)
(-)
(+)
C
R
R
C
C
R
R
C
(+)
(+)
Protein Phosphatases
Substrate Phosphorylation
Substrate Dephosphorylation
Physiological Event

Figure 1.

the effect on phosphatase activity was minimal (Tung, 1986). Although the activities of the protein phosphatases is undoubtably vital for normal steroidogenic function, there has been little attempt to study their functional activity in the Leydig cell.

b) cGMP

A second cyclic nucleotide second messenger system is present in many cell types that is particular interest in the murine Leydig cell: cyclic guanosine 3',5'-monophosphate(cGMP) and its protein kinase (PK-G) (Fig. 1). While the importance of the cAMP messenger cascade has been well established, the prevalence and functional importance of the cGMP system is relatively poorly understood. As previously discussed, atrial natriuretic factor greatly enhanced androgen production in mouse and rat Leydig cells (Pandey et al., Pandey et al., 1986a) via cGMP synthesis (Mukhopadhyay et al., 1986b).

Though similarities exist between the two cyclic nucleotide cascades, there are also some interesting and functionally important differences. Unlike the membrane-bound adenylate cyclase, the guanylate cyclase (GC) enzyme responsible for cGMP generation has been located in the cytoplasm (Kimura and Murad, 1974), cytoskeleton (Fleischman et al., 1980) as well as bound to the plasma

membrane (Chrisman et al., 1975). As this study relates to ANF stimulation of androgen synthesis, only the peptide-stimulated membrane-associated GC will be discussed.

Studies on the particulate GC protein have characterized a number of functionally important domains within the protein sequence. Garbers and Radany (1981) found a lipophilic domain within the peptide sequence which was characteristic of a transmembrane protein. The extracellular domain of GC was found to be almost 34% homologous to the primary amino acid sequence of the low molecular weight ANF receptor (Fuller et al., 1988) and it was proposed that the ANF receptor was actually the membrane-bound form of guanylate cyclase (Garbers, 1989). An investigation on Leydig cell GC activity utilized an antibody raised against an adrenal membrane protein which exhibited both ANF binding capabilities and guanylate cyclase activity (Marala and Sharma, 1988). Exposure to this antibody inhibited ANF-stimulated cGMP generation in the rat and mouse Leydig cell though basal cGMP levels were unaffected. The authors of the study suggested that this species of GC may represent only one of several active isoforms in the Leydig cell. The presence of a G-protein-sensitive species of GC in the Leydig cell has not been demonstrated although evidence in other cell types supports that possibility. Snugden and Klein (1987) showed that cholera toxin enhanced both basal and phenylephrine-

stimulated cGMP content in the rat pinealocyte while Ho et al., (1988) noted that the G_S activator facilitated the stimulatory effects of protein kinase C on cGMP generation in the same cell type.

As is the case for cAMP, activation of cellular processes occurs via a specific phosphotransferase, PK-G (Kuo and Greengard, 1970). Again, the PK-G enzyme has 4 cyclic nucleotide binding sites per kinase , 2 per regulatory domain (MacKenzie, 1982; Corbin and Doskeland, 1983). As was shown to be the case with the PK-A regulatory domain, the cGMP binding sites also exhibited positive cooperativity (McCune and Gill, 1979). PK-G differed from the cAMP-dependent kinase, however, in that cGMP binding did not result in the dissociation of the catalytic subunits from the regulatory peptides and no decrease in M_r was noted (Lincoln et al., 1977). Phosphorylation substrates for PK-G were found to overlap quite substantially with PK-A and both appear to catalyze phosphorylation of the same residues on the same proteins (Lincoln and Corbin, 1977,1983). Indeed, substrate specificity studies using synthetic peptides found similarities in the recognition sites for protein substrates on the catalytic subunits of PK-A and PK-G (Lincoln and Corbin, 1977). Amino acid sequencing studies subsequently demonstrated homology between PK-G and various sequences on the regulatory and catalytic units of PK-A (Takio et al., 1982). These

similarities in enzymatic characteristics caused speculation that a common ancestral precursor protein existed for the two protein kinases (Lincoln and Corbin, 1977). Vardanis (1980) identified and partially characterized such a protein kinase in the grasshopper. A homodimer, the kinase bound both cAMP and cGMP with similar and relatively high affinities (43 and 25 nM, respectively). The kinase did not change molecular weight upon activation, a characteristic reminiscent of the cGMP-dependent protein kinase. The author of the study and others have suggested that this molecular form of a protein kinase could represent a common ancestral structure for the highly homologous cAMP/cGMP-dependent kinases (Vardanis, 1980; Corbin et al., 1988). The possibility that this type of protein kinase could exist in mammalian cell types such as the mouse Leydig cell is exciting and could represent a potential site of interaction between the two cyclic nucleotide messenger cascades.

Interaction between the two cyclic nucleotide systems has been established in a number of different tissues (Goldberg et al., 1975). In many instances, "crosstalk" between the two messenger systems was found to be adversarial in that the guanosine system opposed cellular activities mediated through the cAMP cascade and vice versa (Goldberg et al., 1975; Kudo and Baird, 1984). An example of this "Yin-Yang" relationship (Goldberg et al., 1975) was

noted in cultured tumour Leydig cell in which ANF stimulated cGMP synthesis but reduced intracellular cAMP levels and attenuated the steroidogenic responsiveness to LH (Pandey et al., 1985). How this inhibition is manifested has not been entirely clarified though cGMP-mediated stimulation of cAMP hydrolysis by type II PDE (Weishaar, 1987) is one possibility.

The potential also exists for a cooperative rather than antagonistic relationship between the two cyclic nucleotide systems. There were findings that cAMP and cGMP acted synergistically in olfactory cilia and pinealocytes (Nakamura and Gold, 1987; Ho et al., 1988). Furman and Tanaka (1989) showed that cAMP interacted with cGMP in the cooperative activation of phototransduction in excised patches from retinal rod outer segments. They hypothesized that this represented a physiologically relevant interplay between cAMP and cGMP. The mechanism by which cAMP and cGMP interact is unknown. Affinity of a single enzyme for both cyclic nucleotides is not without precedence (type I and II PDE) (Weishaar et al., 1985; Weishaar, 1987). However, a number of studies demonstrated that the relative specificities of cAMP and cGMP for their respective protein kinases approached two orders of magnitude (Kuo and Greengard, 1970; Lincoln and Corbin, 1983). This would appear to contraindicate any interaction through either phosphotransferase. However, cAMP and other cyclic

nucleotides which bind with low affinity to PK-G enhanced PK-G autophosphorylation (deJong and Rosen, 1977) which occurred at a much slower rate than did PK-A self-phosphorylation. The physiological relevance of this interaction has not been clearly elucidated. An early study showed that PK-G autophosphorylation had little effect on phosphotransferase activity or binding affinity for cGMP (Foster et al., 1981). However, subsequent studies demonstrated that phosphate incorporation doubled the PK-G V_{MAX} with little or no effect on its affinity for cGMP or its substrate (Hofman et al., 1983).

2. Ionic calcium and calmodulin

The concentration of free ionic calcium (Ca^{2+}) in the extracellular space approaches 10^{-3} M, while the intracellular levels are maintained at a basal level of 10^{-8} - 10^{-9} M. By inducing the entry of ionic calcium down this concentration gradient, certain hormones can use free ionic calcium as a second messenger for stimulus-response coupling and regulation of intracellular processes (Rasmussen et al., 1984). In the Leydig cell, the actions of LH, ANF and GnRH are all mediated, at least in some part, through changes in the levels of intracellular calcium (Lin, 1985; Sullivan and Cooke, 1986; Mukhopadhyay et al., 1989). Janszen et al., (1976) and Hall and coworkers (1981) demonstrated that

Leydig cell response to LH required extracellular Ca^{2+} while inhibition of calcium movement across the cell membrane through the use of Ca2+ channel blockers was shown to attenuate the steroidogenic response to LH and dibutyryl cAMP (Lin et al., 1979; Moger, 1983). Sullivan and Cooke (1986) used an intracellular fluorescent Ca^{2+} chelator to demonstrate that exposure of rat Leydig cells to LH or GnRH elicited an increase in intracellular calcium concentrations. In addition, the levels of free calcium in the cytosol increased following exposure to levels of LH which stimulated androgen production but did not elicit any changes in cAMP synthesis. Similarly, Lin (1985) noted that exposure of rat Leydig cells to a GnRH analogue did not elicit any increase in cAMP levels but did require extracellular calcium. Mukhopadhyay and coworkers (1989) demonstrated that while an absence of extracellular calcium inhibited the steroidogenic response to ANF without affecting cGMP accumulation, an excessive ionophoretic influx of Ca^{2+} inhibited the response to ANF.

Certain agonists may also increase free calcium availability by eliciting the release of intracellular calcium stores. This receptor-mediated elevation in cytosolic calcium levels occurs via hydrolysis of the minor plasma membrane constituent phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DG) by the membrane-bound enzyme,

phospholipase C (PLC; for review, see Berridge and Irvine, 1984 and Fig. 2). Interaction between the receptor and PLC is not direct but instead occurs via G-proteins (as discussed above; Snyderman et al., 1986). Upon their release, IP_3 and DG synergistically mediate signal transduction in receptor systems linked to calcium mobilization. IP_3 acts to release sequestered Ca^{2+} from intracellular stores such as the endoplasmic reticulum (Rasmussen et al., 1984) while DG activates the calcium/phospholipid-dependent phosphotransferase, PK-C (Huang et al., 1988). Although it could be argued that IP_3 and DG are the true second messengers, this literature review will concentrate on the role of ionic calcium and its binding protein, calmodulin, in the control of cellular activities. Again, points of interaction with the other second messenger systems will be highlighted.

a) Calmodulin

Calcium influences intracellular events both directly as an enzymatic cofactor and indirectly via binding proteins. Review of the literature revealed that while a large number of calcium-regulated proteins have been identified, their functional significance and tissue distribution has not been well established (Manalan and Klee, 1984). One Ca^{2+}-binding protein which has been

Fig. 2. The calcium/calmodulin second messenger cascade. This figure schematically illustrates the mechanisms by which receptor/G protein complexes can initiate increases in the levels of intracellular ionic calcium. Note the phospholipase C (PLC)-mediated release of inositol triphosphate (IP_3) and diacylglycerol (DG) and how these factors act to elicit the release on intracellular Ca^{2+} stores and activation of protein kinase C (PK-C). The influence of Ca^{2+} and its binding protein, calmodulin (CaM) on substrate phosphorylation via PK-C and other phosphotransferases and protein phosphatases is also illustrated. (+) represents a stimulatory influence.

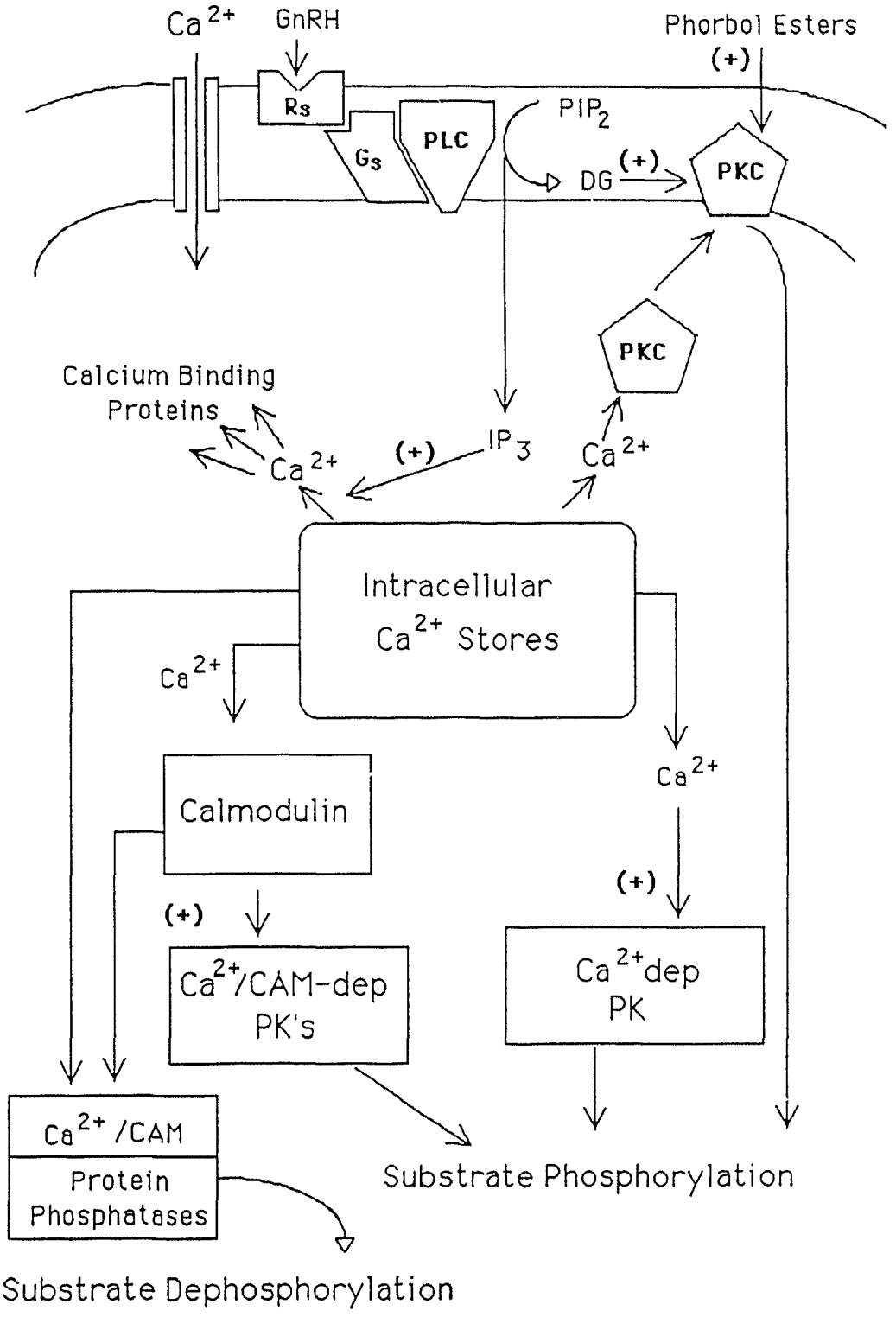

Ca 2+
GnRH
Phorbol Esters
(+)
Rs
PLC
Gs
PIP2
DG
(+)
PKC
PKC
Calcium Binding
Proteins
Ca 2+
(+)
IP3
Ca 2+
Intracellular
Ca 2+ Stores
Ca 2+
Ca 2+
Calmodulin
(+)
(+)
Ca 2+/CAM-dep
PK's
Ca 2+dep
PK
Ca 2+ /CAM
Protein
Phosphatases
Substrate Phosphorylation
Substrate Dephosphorylation

Figure 2.

extensively studied is calmodulin (CaM) (Manalan and Klee, 1984; Means, 1988; Persechini et al., 1989). This ubiquitous protein ($M_r \approx 17$ kD) was shown to contain four helical calcium binding sites, or "EF hands". These Ca2+-binding sequences, which bind calcium with dissociation constants of approximately 1 μM (Kilhoffer et al., 1983), have also beenlocated in other Ca2+ modulated peptides such as troponin C, S-100 and parvalbumin (Persechini et al., 1989). The affinity of the EF hands for ionic calcium suggest that CaM can be modulated by the range of calcium concentrations found inside the cell. Occupation of a minimum of two of these binding sites was sufficient to initiate the bulk of the critical conformational changes (Klevit et al., 1984), exposing the hydrophobic domain which allows CaM to form a high affinity 1:1 complex with the target protein (LaPorte et al., 1980). In this manner, calmodulin acts to translate fluctuations in intracellular calcium levels into functional alterations in the activity of a number of different enzyme systems (Means, 1988) including adenylate cyclase (Sharp et al., 1980; Wolff et al., 1980), myosin light chain kinase (Blumenthal and Stull, 1982), cyclic 3',5'-nucleotide phosphodiesterase (Cheung, 1970), Ca2+/calmodulin-dependent protein kinase (Nairn, Hemmings and Greengard, 1985) and possibly phospholipase A_2 (Wong and Cheung, 1979; Craven and DeRubertis, 1985; Nakagawa and Waku, 1988).

A number of calmodulin antagonists have been

developed to study the multifunctional aspects of calmodulin's role in the cell. Although encompassing a diversity of structural types, the mode of action of these antagonists almost invariably involves interaction with the hydrophobic substrate binding domain exposed upon calcium binding to calmodulin (Manalan and Klee, 1984). The antipsychotic phenothiazine drug, trifluoperazine (TFP), was found to inhibit CaM-dependent phosphodiesterase activity (Levin and Weiss, 1977) and as a result, has been used extensively as a calmodulin antagonist (Wong and Cheung, 1979; Hall et al., 1981; Hait and Lee, 1985). It soon became apparent, however, that the questionable specificity and cytotoxic effects of TFP (Wrenn et al., 1981; Hait and Lee, 1985; MacNeil et al., 1988) made it less than optimal for CaM studies. The original investigation on the role of CaM in Leydig cell function showed that the introduction of Ca^{2+}/CaM into Leydig cells with liposomes augmented the delivery of cholesterol to the inner mitochondrial membranes and increased testosterone production (Hall et al., 1981). This effect could be abolished with concurrent exposure to 50 μM TFP. While the effect of CaM on steroidogenesis is not in dispute, the level of TFP utilized in this study was more than an order of magnitude above its IC_{50} for calmodulin-mediated events and approached the IC_{50} of the drug for protein kinase C (74 μM; MacNeil et al., 1988). Therefore, while some of the effects of TFP in the study by

Hall et al. (1981) certainly derived from specific CaM antagonism, the possibility of nonspecific actions must be addressed.

The napthalenesulfonamide CaM antagonist, N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W7) was subsequently found to be somewhat more specific for calmodulin than TFP (Tanaka et al., 1982; MacNeil et al., 1988) while its lipophilic nature allowed for better access to the intracellular environment than did the anti-calmodulin peptides mellittin (Katoh et al., 1982) and seminalplasmin (Gietzen and Galla, 1985) or other hydrophilic antagonists such as calmidazolium (Mazzei et al., 1984). The availability of the much less potent dechlorinated analog, W5 (Hidaka et al., 1981) also provided a means to assess the calmodulin specificity of any W7 response.

Examination of the literature pertaining to the characteristics of these antagonists provides a reminder regarding the pitfalls associated with the reliance on the use of pharmacological agents for the study of physiology. The literature is full of revelations regarding the lack of specificity, high degree of cytotoxicity and generally unforseen inadequacies of these wonder drugs, usually a number of years and dozens of studies after their introduction into the research world (Franson et al., 1980; Lanni and Becker, 1985; Hait and Lee, 1985; Watanabe et al.,

1986; MacNeil et al., 1988; Brumley and Wallace, 1989; Ruegg and Burgess, 1989). This is not to say that these agents are not important tools in the study of cellular physiology, only that caution should be used in drawing any universal conclusions based on data obtained through their use. The development of new strategies for the manipulation of intracellular activities, such as the use of antisense oligonucleotides as pharmaceutical agents (Cohen, 1989), will allow for much greater selectivity.

b) Protein kinase C

The activities of a number of enzymes within the messenger cascades has been shown to be dependent on Ca^{2+} for optimal activity, including the aforementioned protein kinase C. Purified from the brain by Inoue and coworkers (1977), PK-C was initially characterized as a Ca2+/DG/phospholipid-dependent kinase (Kishomoto et al., 1980). Originally it was thought that a rise in intracellular calcium levels promoted the binding of Ca2+ to cytosolic PK-C, triggering its insertion into the lipid plasma membrane and subsequent interaction with acidic phospholipids such as phosphatidlyserine. The membrane-bound form of PK-C was then activated by diacylglycerol (DG) derived from phospholipase C-catalyzed breakdown of PIP_2 or phosphatidylcholine. In other words, ionic calcium

facilitated the translocation of the cytosolic kinase to the membrane wherein it was stabilized and activated by DG (Huang, 1989).

In fact, this model was only partially accurate. It would appear that the PK-C enzymes comprise a family of structurally related phosphotransferases which vary in their dependence on ionic calcium for activation (Huang, 1989; Parker et al., 1989). The "classical" Ca2+/phospholipid-dependent PK-C subtype contains three isotypes (α/I, β/II and γ/III) and is referred to as the Group A PK-Cs. The Group B subtypes (δ, ϵ and Γ) differ from the Group A kinases in that their activity, while dependent on phospholipids and DG, is independent of Ca2+ (Schaap et al., 1989). The presence of Ca^{2+}-independent PK-C isoenzymes increases the flexibility of the cellular response by giving the cell more options in the way it responds to different agents (Parker et al., 1989). Phospholipase C can generate diacylglycerol by the hydrolysis of a wide variety of membrane phospholipids including phosphatidylcholine (Bockino, Blackmore and Exton, 1985). Therefore, the cell has the ability to generate DG without any concurrent release of IP_3 and subsequent mobilization of intracellular calcium.

The involvement of protein kinase C has been demonstrated in the control of steroidogenic function in Leydig cells (Mukhopadhyay et al., 1984; Lin, 1985; Themmen

et al., 1986; Inoue and Rebois, 1989) as well as in other steroidogenic tissues (Culty et al., 1984; Hofeditz et al., 1988). Exposure of Leydig cells to phorbol esters which act as a DG substitute (Castagna et al., 1982) stimulated basal androgen production in mouse and rat Leydig cells (Mukhopadhyay et al., 1984; Themmen et al., 1986). The mechanism through which PK-C enhanced steroidogenesis has not been fully elucidated but may involve sterol carrier protein$_2$ (SCP_2), the factor responsible for delivery of cholesterol to the mitochondrial P450scc in steroidogenic tissues (Chanderbhan et al., 1982) including the Leydig cell (Hall et al., 1981). Incubation of rat Leydig cells with phorbol esters resulted in a two-fold increase in androgen production and a parallel doubling of SCP_2 levels (van Noort et al., 1988). In addition, stimulatory effects of LH on SCP_2 levels were found to be dependent on the presence of extracellular Ca^{2+} and could be abolished with calcium channel blockers (van Noort et al., 1988). Steinschneider and coworkers (1989) demonstrated that PK-C phosphorylated SCP_2 purified from rat liver while other Ca^{2+}-dependent and -independent kinases could not use the carrier as a substrate. The effect of this phosphorylation on the transport capabilities of SCP_2 was not established although increased intracellular Ca^{2+} levels increased delivery of the sterol to the inner mitochondrial membranes (Hall et al., 1981).

A number of studies have found that PK-C activation may enhance or diminish the activity of hormone-responsive adenylate cyclase (Nishikuza, 1986). In the mouse Leydig cell, PK-C activation reduced cAMP accumulation and androgen production in response to gonadotropin stimulation (Mukhopadhyay et al., 1984; Mukhopadhyay and Schumacher, 1985) while inhibiting the steroidogenic response to submaximal levels of LH (but not to cAMP) in the rat (Themmen et al., 1986). Enhancement of cAMP degradation by PK-C was not a factor in this response as concurrent PDE inhibition did attenuate the reduction of cAMP accumulation following exposure to the phorbol esters (Mukhopadhyay and Schumacher, 1985). The steroidogenic response to ANF in the mouse Leydig cell was also diminished upon PK-C activation though the response to 8-bromo cGMP was not effected (Mukhopadhyay and Leidenberger, 1988).

The lack of an inhibitory effect on the cellular response to the cyclic nucleotide analogues suggested that the site of PK-C influence was at a point prior to messenger generation. Indeed, a number of studies have suggested that activation of PK-C interfered with the interaction between LH-sensitive adenylate cyclase and its G proteins. It was found that treatment of tumour or freshly isolated Leydig cells with phorbol esters induced desensitization of adenylate cyclase to gonadotropin stimulation in a manner akin to that observed following prolonged exposure to LH

(Mukhopadhyay and Schumacher, 1985; Themmen et al., 1986; Rebois and Patel, 1985). The effect appeared to be specific to PK-C stimulation as direct addition of the tumour promoters to purified membrane preparations from untreated Leydig cells did not inhibit AC activity (Mukhopadhyay and Schumacher, 1985). Unlike the LH-induced desensitization, however, exposure to phorbol esters enhanced the ability of cholera toxin to stimulate cAMP generation, an effect which was not potentiated by pertussis toxin-mediated inhibition of G_i activity (Platts et al., 1988). This suggested that differences in AC desensitization elicited by phorbol esters and that arising from LH exposure resided at the G-protein level. Platts and coworkers (1988) proposed that phorbol esters inhibited the function of both G_s and G_i which would explain the lack of sensitivity of these cells to further G_i inhibition by pertussis toxin. Desensitization mediated by LH involved only interference with G_s activation of adenylate cyclase while sensitivity to G_i inhibition remained intact.

A number of Ca^{2+}/CaM-dependent phosphotransferases have been identified. Some, such as myosin light-chain kinase, phosphorylase kinase and the Ca^{2+}/calmodulin-dependent protein kinases have been extensively studied. In most cases, however, the enzymatic properties and tissue distribution of these kinases have not been well characterized (for review, see Manalan and Klee, 1984). In

all cases, their functional significance in the control of Leydig cell steroidogenic activities remains to be elucidated.

c) Interaction between Ca^{2+}/calmodulin and the cyclic nucleotide second messenger systems

A number of other enzymes within the second messenger cascades have been shown to be calcium-dependent including neuronal adenylate cyclase, guanylate cyclase, cAMP/cGMP-dependent phosphodiesterase and a number of the oxygenases within the eicosanoid cascade (which will be discussed in the next section).

Ca^{2+}/CaM stimulated adenylate cyclase in the brain (Wolff et al., 1980), the Islets of Langerhans (Sharp et al., 1980) and other cells (Manalan and Klee, 1984). However, ionic calcium has also been shown to inhibit adenylate cyclase activity in certain other cells (Dorflinger et al., 1984; Giannattasio et al., 1987). For example, Peirera and coworkers (1988) noted that treatment of Leydig tumour cell with calcium ionophores reduced hCG-stimulated accumulation of cAMP and progesterone without any effect on the rate of cAMP degradation. In the absence of the ionophores, however, the level of extracellular Ca^{2+} did not influence the rate of synthesis nor the total accumulation of cAMP in basal and hCG-stimulated cells. The

physiological relevance of these ionophore studies to normal Leydig cells could be questioned due to the nature of the experimental model (tumour-derived cells) and the probability that the degree of intracellular access to extracellular calcium ions facilitated by the ionophores could be excessive.

Indeed, there has been evidence suggesting that the influence of ionic calcium of cAMP generation was variable. Dufau et al. (1987) demonstrated that low concentrations of ionic calcium stimulated adenylate cyclase activity in purified rat Leydig cell membranes while higher Ca^{2+} levels were inhibitory. Although the relevance of these findings to the intact cell was not addressed (Dufau et al., 1987), review of the literature suggests that Ca^{2+} could also have a biphasic influence on AC activity in situ. Dufau et al. (1987) showed that nanomolar Ca^{2+} concentrations enhanced LH-stimulated cAMP synthesis over that seen in calcium-free conditions. This concentration of calcium was equivalent to that found in the cytoplasm unstimulated rat Leydig cells (1-10 nM) in studies with intracellular calcium indicators (Sullivan and Cooke, 1986). The concentrations of Ca^{2+} shown to inhibit cAMP generation in response to LH ($\geq$ 80 nM; Dufau et al., 1987) were equivalent to the levels of ionic calcium measured in LH-stimulated Leydig cells (Sullivan and Cooke, 1986). The effect of Ca^{2+} was mediated through changes in the number of AC units activated by the gonadotropin rather

than the affinity of the enzyme for the LH receptor as no significant change in EC_{50} for LH was noted (Dufau et al., 1987). These findings suggest the presence of a negative feedback loop operating in the Leydig cell in which basal intracellular calcium concentrations facilitate LH stimulation of AC. The subsequent LH-mediated influx of Ca^{2+} into the intracellular environment then acts to decrease responsiveness to the gonadotropin, thereby controlling the response.

A definitive role for Ca2+ in determining GC activity has not been established. Ionic calcium was identified as an important modulator of cGMP metabolism in a number of the earlier studies on this messenger system (Schultz et al., 1973; Goldberg and Haddox, 1977). Subsequent research noted that the influence of ionic calcium varied with the cell type. For example, work with membrane preparations from Paramecium and Tetrahymena demonstrated a stimulatory role for Ca2+ which appeared to be mediated through calmodulin, at least in the Paramecium (Klumpp and Schultz, 1982; Klumpp et al., 1982). Alternatively, studies with the outer segments of retinal rods suggested a direct inhibitory effect of ionic calcium on GC activity (Fleischman et al., 1980), again mediated by an intermediary protein (Koch and Stryer, 1988). The effect of ionic calcium on cGMP generation in the mouse Leydig cell has not been completely elucidated. Although the removal of extracellular calcium

had no effect on the accumulation of cGMP in response to ANF, the steroidogenic effect was inhibited by 50% (Mukhopadhyay et al., 1989). On the other hand, co-exposure to ANF and a calcium ionophore inhibited ANF-stimulated cGMP generation although guanylate cyclase isolated from the mouse Leydig cells was completely insensitive to both Ca^{2+} and CaM antagonists. These findings would suggest that Ca^{2+} does not directly inhibit GC activity but may do so indirectly, possibly via an inhibitory PK-C system in the intact cell. Activation of the Ca^{2+}-dependent phosphotransferase with phorbol esters has been shown to markedly inhibit the accumulation of cGMP in response to ANF in the mouse Leydig cell (Mukhopadhyay and Leidenberger, 1988) and other cell types (Jaiswal et al., 1988).

While its influence on the generation of cyclic nucleotide messengers has yet to be resolved, the effect of Ca^{2+} on cAMP/cGMP degradation has been more fully characterized. A number of different species of phosphodiesterase enzyme with varying sensitivities to intracellular calcium have been identified. The activity of type I PDE, which will hydrolyze cAMP and cGMP to comparable degrees was stimulated two- to three-fold by CaM in the presence of calcium while the type II and III isoforms were insensitive to changes in CaM/Ca2+ availability (Weishaar, 1987).

3. The eicosanoid cascade

a) Arachidonic acid

The eicosanoids are bioactive metabolites formed by oxidation of arachidonic acid (AA) (Fig. 3). AA can be generated either directly, by the action of phospholipase A_2 (PLA_2) on a variety of membrane phospholipids (Michel, 1975) or indirectly, through the action of diglyceride lipase on diglycerides liberated by the prior action of phospholipase C on phosphotidylinositides (Irvine, 1982) or phosphatidylcholine (Loeb and Gross, 1986). Alternatively, these diglycerides can be phosphorylated by diglyceride kinase to form phosphatidic acid and then used as a substrate for the PLA_2 enzyme (Loeb and Gross, 1986). It was established that the release of AA by PLA_2 was mediated via both G_s- and G_i-protein transducers in a number of different cell types (Samuelsson et al., 1978; Axelrod, Burch and Jelsema, 1988). This determination was based on the sensitivity of the system to exogenous GTP or the GTP analogues, GTPγS or GppNHp.

Fig. 3. The eicosanoid second messenger cascade. The influence of receptor/G protein complexes on the generation of arachidonic acid (AA) by phospholipase A_2 (PLA_2) is illustrated. Note the potential for AA oxidation by cyclooxygenase, lipoxygenase and epoxygenase enzymes and the role of Ca^{2+}/CaM in controlling the activities of these enzymes. (+) represents a stimulatory influence while (?) indicates putative involvement.

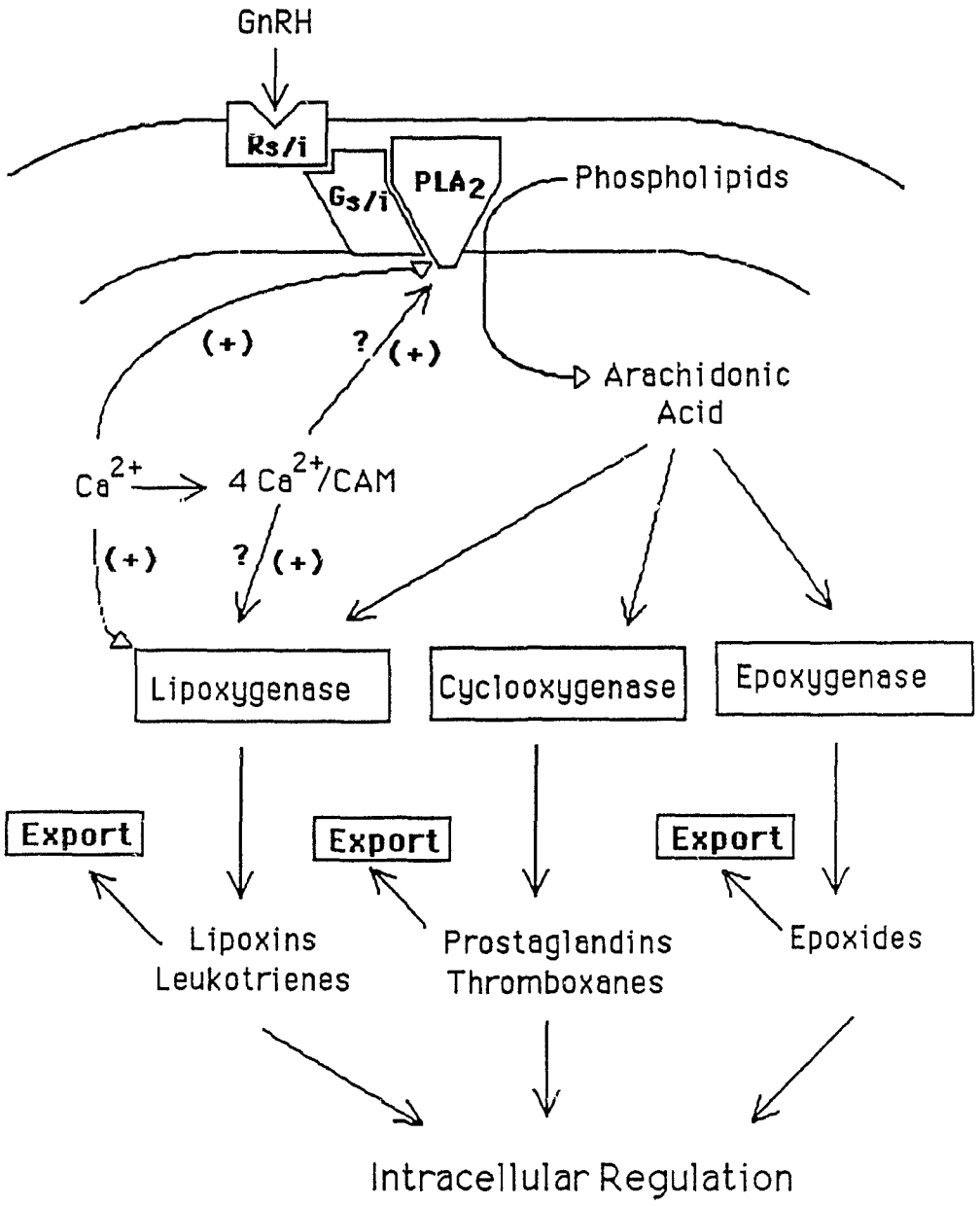

GnRH
Rs/i
Gs/i
PLA2
Phospholipids
(+)
?
(+)
Arachidonic Acid
Ca2+
4 Ca2+/CAM
(+)
?
(+)
Lipoxygenase
Cyclooxygenase
Epoxygenase
Export
Export
Export
Lipoxins
Leukotrienes
Prostaglandins
Thromboxanes
Epoxides
Intracellular Regulation

Figure 3.

b) The eicosanoids and their interactions with other second messenger systems

Oxidation of AA has been shown to occur via three major enzymatic pathways. The prostaglandin endoperoxide synthase enzyme, which encompasses both cyclooxygenase and peroxidase activities (Samuelsson et al., 1978), acts as a catalyst in the synthesis of prostaglandins and thromboxanes. The lipoxygenases utilize AA to generate leukotrienes (by 5-lipoxygenase) and lipoxins (by 15-lipoxygenase) while the cytochrome P450 epoxygenases catalyze the formation of epoxides (for review, see Needleman et al., 1986). Arachidonic acid and its eicosanoid derivatives are interesting in that they can act extracellularly as autocrine/paracrine factors and as intracellular second messengers (Samuelsson et al., 1978). This review will concentrate on their intracellular regulation and actions.

The eicosanoid cascade has been implicated as an intracellular mediator in a number of physiological effectors including angiotensin II, bradykinin, prolactin and interleukin 1 (Schartzmann and Raz, 1979; Rillema and Wild, 1977; Chang, Gilman and Lewis, 1986) and in the stimulation of Leydig cells by GnRH and LH (Lin, 1985; Abayasekara, Band and Cooke, 1990). The mechanisms by which AA and its eicosanoid metabolites influence intracellular

events has not been well characterized. Rather than directly activating a specific protein kinase, the eicosanoids seem to indirectly modulate cellular events through interaction with the cyclic nucleotide and calcium messenger cascades.

Interactions with cAMP/cGMP system have been noted at several levels ranging from the point of synthesis through the time of degradation. Endoperoxides formed during AA metabolism activated soluble guanylate cyclase (Goldberg and Haddox, 1977) while other prostaglandins (PG) have been implicated in the direct stimulation of GTPase activity of the $G_S\alpha$ subunit thereby reducing adenylate cyclase activity (Bitonti et al., 1980). Alternatively, the rate of cyclic nucleotide hydrolysis may also be augmented in that lysophosphotidylcholine generated by PLA_2 enhanced PDE activity (Needleman et al., 1986). PLA_2 activity may, in turn, be influenced by cAMP (Samuelsson et al., 1978) as dibutyryl-cAMP decreased levels of free AA in human platelets (Minkes et al., 1977).

The role of the eicosanoid cascade in Leydig cell function is unclear. While exogenous arachidonic acid or PLA_2 derived from snake venom enhanced androgen production by the rat Leydig cell (Lin, 1985; Abayasekara et al., 1990), basal testosterone production was unaffected by PLA_2 inhibitors (Abayasekara et al., 1990). The steroidogenic response to LH, dibutyryl-cAMP and forskolin was reduced by the inhibitors, however (Abayasekara et al., 1990). The

stimulatory effect of AA was significantly enhanced by PDE inhibition suggesting the involvement of cyclic nucleotides in the response (Lin, 1985). However, as cAMP synthesis was not affected by PLA_2 inhibition, any eicosanoid-mediated support of cAMP-mediated cellular activities must occur beyond the point of synthesis (Abayasekara et al., 1990). The effect of PDE inhibition with MIX noted by Lin (1985) may not be mediated via cAMP, however; events mediated by cGMP would also be potentiated by any reduction in phosphodiesterase activity (Mukhopadhyay et al., 1986a). The potential for interaction between the eicosanoid cascade and cGMP in the Leydig cell was not addressed.

Events mediated by the eicosanoid cascade are indirectly influenced through interaction with the Ca^{2+}/CaM messenger system. Several enzymes in eicosanoid synthesis have been shown to be calcium-dependent including phospholipase A_2 (Rittenhouse-Simmons and Deykin, 1977; Jesse and Franson, 1979) and the 5-lipoxygenase enzyme (Jakschik et al., 1980; Rouzer et al., 1985). Increasing Ca2+ availability with an ionophore enhanced PLA_2 activity (Rittenhouse-Simmons and Deykin, 1977) while chelation of intracellular calcium inhibited AA release (Simon et al., 1986). There is evidence that a calcium-binding site actually resides within the protein structure of the PLA_2 enzyme (van Scharrenburg et al., 1985).

There is a possibility that some of these calcium-

sensitive enzymes may also require calmodulin for optimal activity. As was the case for purified membrane-bound PLA_2 (Jesse and Franson, 1979; Nakashima et al.,1989), abnormally high levels of Ca^{2+} were required for optimal activation of the lipoxygenase enzymes <u>in vitro</u> ($ED_{50} \approx 0.1-1.0$ mM) (Rouzer et al., 1985). This prompted Shimizu (1988) to suggest that a factor may be present <u>in situ</u> which acts as an intermediary to more efficiently couple the lipoxygenase enzyme with the calcium ion. Calmodulin, as a calcium binding protein, could act in this capacity as may or may not be the case for the PLA_2 enzyme (Wong and Cheung, 1979; Withnall et al., 1984; Craven and DeRubertis, 1985; Watanabe et al., 1986; Nakagawa and Waku, 1988)

Arachidonic acid could also influence Leydig cell function in a manner independent of its role as an eicosanoid substrate. Accumulation of AA following the inhibition of the lipoxygenase enzyme with nordihydroguaiaretic acid (NDGA) resulted in the stimulation of androgen production in the rat Leydig cell (Dix et al., 1984; Lin, 1985; Didolkar and Sundaram, 1989). Lin (1985) demonstrated that the stimulation of androgen production upon exposure to exogenous AA was potentiated by concurrent inhibition of the cyclooxygenase and lipoxygenase enzymes suggesting that arachidonic acid alone was a stimulatory factor in the Leydig cell. How arachidonic acid stimulates the steroidogenic function of Leydig cells is unclear but

may well be mediated via interaction with the Ca2+ second messenger system. Free AA enhanced the activity of phospholipase C (Zeiller and Handwerger, 1985) which has been shown to stimulate androgen production by rat Leydig cells (Didolkar and Sundaram, 1989). Exogenous AA was also found to stimulate a rise in cytosolic calcium levels independent of inositol triphosphate release (Wolfe et al., 1986). McPhail and coworkers (1984) demonstrated that AA stimulated PK-C in a calcium-dependent manner which was enhanced by diolein but did not require exogenous phosphatidylserine. Lin (1985) subsequently showed exogenous AA to both stimulate PK-C and to augment its activation by ionic calcium and phospholipids in rat Leydig cells. Naor et al. (1988) more fully evaluated the role of arachidonic acid in PK-C activation, noting that γ-subtype was more sensitive to AA stimulation than were either the β- or α-PK-C isotypes. Although it has not been established which PK-C subtype is dominant in the Leydig cell, stimulation of PK-C by AA could, at least in part, mediate the inhibitory effect of NDGA on gonadotropin-stimulated androgen production (Dix et al., 1984; Lin, 1985).

The goal of the studies in this thesis is to more fully characterize the role of various second messenger networks in the control of androgen production by the mouse Leydig cell. The potential for interaction between intracellular regulators will be addressed and the

functional significance of these interactions in the regulation of testicular androgen production will be assessed. Specifically, the role of the two major PK-A isoenzymes in the regulation of steroidogenesis will be examined as will the possibility that a synergistic interaction between the cAMP and cGMP systems may be involved in the hormonal support of testicular androgen production. Lastly, experimental evidence will be presented which supports the presence of an inhibitory calmodulin-sensitive lipoxygenase enzyme in the mouse Leydig cell.

II. MATERIALS AND METHODS

A. Materials

The calmodulin antagonist, N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W7) and its dechlorinated analog, N-(6-aminohexyl)-1-napthalenesulfonamide (W5) were procured from Sigma Chemicals (St. Louis, Mo.) as were aminohexylamino- (AHA), N^6-benzoyl- (B) and 8-thiomethyl (TM)-cAMP, cAMP, 8-bromo-cGMP, cGMP, forskolin, ANF (rat atriopetin II), tissue culture medium-199 (TC-199), $NaHCO_3$, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), L-glutamine, pyruvate, penicillin/streptomycin, 3-isobutyl-1-methylxanthine (MIX) and bovine serum albumin (BSA; fraction V). Percoll and dextran T-70 were procured from Pharmacia Fine Chemicals (Dorval, P.Q.) while Bio-gel P2 (200-400 mesh) was obtained from Bio-rad Laboratories (Richmond, CA.). Collagenase derived from Clostridium hystolyticum (143 U/mg) was purchased from Boehringer Mannheim (Laval, P.Q.). NIH-LH-B9 (0.7 X NIH-LH-S1) was a generous gift from Dr. L. Reichert, Jr. and the NIAMDD Pituitary Hormone Distribution Program. Activated charcoal powder was obtained from BDH Chemicals (Toronto, Ont.) while testosterone for the androgen assays was purchased from Steraloids, Inc. (Whilton, NH) and stored in ethanol (100 ng/ml) at 4°C. All other chemicals were purchased from

Fisher Chemical Co. (Dartmouth, N.S.) unless otherwise stated.

[1,2,6,7-^{3}H]Testosterone ([^{3}H]T; specific activity 92 Ci/mmol) utilized in androgen radioimmunoassays was purchased from Amersham (Oakville, Ont.) and stored as a stock solution (4.4 X 10^{7} DPM/ml) in benzene/absolute ethanol (9:1 vol/vol) at 4°C. ^{125}I-cyclic nucleotides (≈ 1 μCi ^{125}I-succinyl cGMP-tyrosine methyl ester and ≈ 1.5 μCi ^{125}I-succinyl cAMP-tyrosine methyl ester were acquired as part of a radioimmunoassay (RIA) kit (Biomedical Technologies Inc., Stoughton, MA). The [^{125}I]cAMP and [^{125}I]cGMP were dissolved in sodium acetate buffer and stored at 4°C for a maximum of 2 months.

B. Animals

Adult male Swiss Webster mice (25-30g) were obtained from Charles River Breeding Laboratories (Kingston, MI) and housed five per plastic cage (28 X 19 X 13 cm) in the Animal Care Centre, Dalhousie University.

C. Buffers and culture media

All cell incubation and perifusion experiments in this study utilized tissue culture medium 199 (TC-199) which was prepared by dissolving 9.8 g of dried medium in

approximately 1 litre of distilled, deionized water. The media was further augmented with 2.22 g sodium bicarbonate (27mM), 2.38 g HEPES (10mM), 100 mg L-glutamine (0.7mM) and 110 mg pyruvate (1mM). The media solution was then adjusted to pH 7.4 with 5N HCl, brought up to 1 L in volume and filter sterilized by positive pressure through a 0.22 μm filter (Milipore Corp., Bedford, MA). BSA was added to the media (1 mg BSA/ml TC-199) immediately prior to use, unless otherwise stated.

A ten-fold concentrated HEPES (10 X HEPES) solution was used to prepare isotonic Percoll (90% Percoll, 10% 10 X HEPES) for the linear density gradients. It contained 100 mM HEPES, 50 mM glucose, 1.45 M NaCl, 50 mM KCl and 10 mM $CaCl_2$, pH 7.4.

Steroid assays utilized a 0.1 M phosphate buffer consisting of 154 mM NaCl, 0.1% gelatin, 61.4 mM Na_2HPO_4, 38.6 mM $Na_2HPO_4.H_2O$ and 0.25 mM thimerosal (ethylmercurithiosalicyclate) in distilled water, pH 6.8. The steroid buffer was stored at 4°C and used within 3 weeks of preparation.

D. Testicular dispersion and purification of Leydig cells

1. Dispersion of mouse testis

Mouse Leydig cells were prepared by the enzymatic method previously described by Moger and Murphy (1983). Briefly, adult male mice were sacrificed by carbon dioxide asphixiation and castrated. The testes were decapsulated and put into 50 ml conical tubes containing an enzyme solution (1 ml/testes) consisting of 0.33 mg Type-1 collagenase and 10 mg BSA per millilitre TC-199. A maximum of 18 testes were dispersed in one 50 ml tube. The tubes were capped and placed on their sides in a heated water bath (34°C) oscillating at 80 cycles/min for approximately 15 min. After incubation, the enzymatic processes were stopped by adding 20-30 ml of cold TC-199 to the testicular preparation. The tubular elements were allowed to sediment out of solution by placing the tubes upright in an ice bucket for 5 min. The supernatent was then decanted into another 50 ml tube and poured into another tube through nylon mesh (72 and 44 μm pore size), thereby removing any remaining tubule fragments. The interstitial cells were pelleted by centrifugation (120 X g for 5 min) in a Clinical centrifuge (International Equipment Co., Boston, Massachusetts, dial set at #4). The cells were then resuspended in TC-199 prior to gradient purification.

2. Leydig cell purification

The interstitial cell preparation was purified through a 0-90% Percoll gradient as previously described by Moger and Murphy (1983) with small modifications. The gradients were prepared using a gradient former (Chrismac Plastic Fabrications, Canada) which gradually mixed equal volumes (20 ml) of TC-199 and isotonic Percoll (Percoll:10 X HEPES, 9:1). The gradient former consisted of two chambers connected by a channel which could be opened or closed with a valve. A length of flexible tubing was inserted into the bottom of the right chamber which contained isotonic Percoll and a magnetic flea for stirring. TC-199 was put into a chamber on the left side of the gradient former. The mixing process was initiated by opening the valve between the two chambers. By simultaneously placing the opposite end of the flexible tubing into a 50 ml conical tube located below the gradient former, undiluted Percoll flowed out of the Percoll chamber and into the tube. As this occurred, TC-199 flowed into the chamber and was mixed with the Percoll by the magnetic flea. In this manner, the Percoll solution was progressively diluted before it flowed into the 50 ml tube and a linear density gradient was formed.

Interstital cells resuspended in TC-199 were layered on the top of the Percoll gradients (6-8 testes/2

ml/gradient) and spun for 15 min at 900 X g in a refrigerated PR-6000 centrifuge (Damon/IEC Division, Needham Hts., Mass.). The Leydig cell layer (located between the 15-25 ml markings on the 50 ml tube) was removed with a 35 ml syringe and a 7.4 cm long 16 guage needle (Critikon Canada Inc., Markham, Ont.), diluted with TC-199 (1:3 v/v) and centrifuged at 120 X g for 5 min. The cell pellet was washed three times with TC-199, resuspended in 2ml of TC-199 and the Leydig cell number assessed using a hemacytometer.

E. Leydig cell incubations

Leydig cells (20,000 cells/500μl final volume of TC-199) were incubated in 12 X 75 mm polystyrene, round bottomed tubes for 3h. A warm (34°C), oxygenated (95% O_2,5% CO_2) and humidified atmosphere was maintained with a shaking water bath (80 oscillations/min). Chemical mediators were diluted such that the desired treatment levels could be attained with additions of 10μl/tube. The incubations were terminated by centrifugation (1000 X g for 5 min) in a refrigerated centrifuge (4°C). The supernatent was then decanted and stored at -20°C in capped sample pots until assayed for androgens or cyclic nucleotides.

F. Leydig cell perifusions

1. The perifusion chamber

One of the perifusion chambers utilized in these experiments is illustrated intact (Fig. 4) and disassembled (Fig. 5). These chambers were constructed by the National Institute of Health for Dr R. Rittmaster (Dalhousie University) who has kindly allowed their use in this study. The chamber barrel (Fig 5F) was a clear plastic cylinder (10 mm I.D., 25.4 mm O.D.) with an internal volume of approximately 1.3 ml. A mounting bracket was attached to the side of the chamber barrel. The top and bottom of the chamber barrel were machined out to accept the insertion of a nylon filter disc (5 μm pore size, 10 mm diameter, Fig. 5E and G), held in place by a fritted plastic disc (10 mm diameter, Fig. 5D and H). The filter and disc ensured that the cells and support matrix are contained within the interior of the chamber barrel. The exterior surface on both ends of the chamber barrel had also been machined to thread into the chamber cap (Fig. 5B and J) which was the female inlet of a 13 mm Swinnex disc filter holder containing a silicone gasket (Fig. 5C and I) to prevent leakage (Millipore Corp., Bedford, MA). A two-way stopcock (Fig. 5K) inserted into the side of the chamber barrel acted as an injection port for the introduction of the cells into the

Fig. 4. A cell perifusion chamber and bubble trap used for the perifusion experiments. Note the presence of a 8 cm ruler for scale.

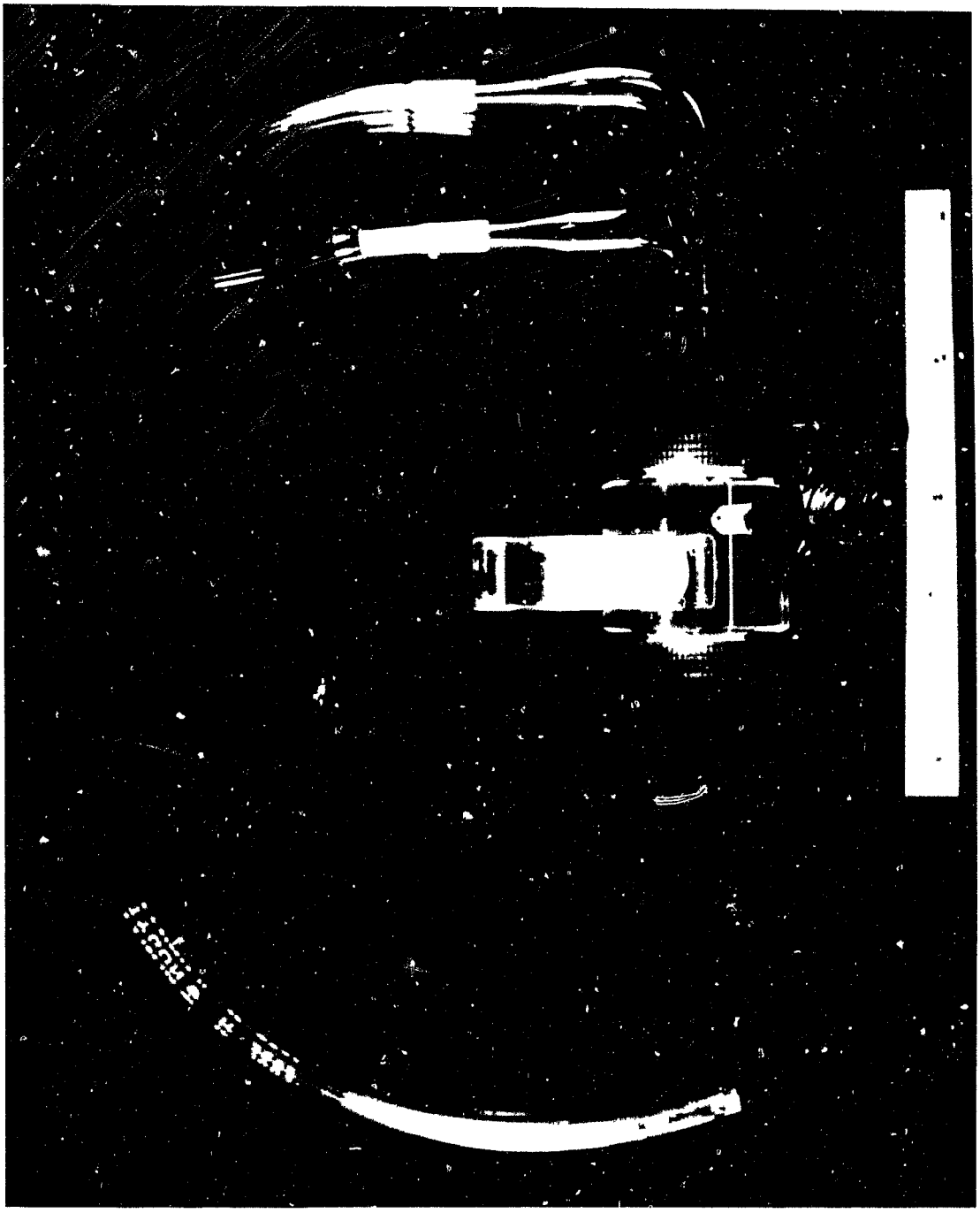


Figure 4.

Fig. 5. The component parts of a perifusion chamber including a bubble trap. This figure illustrates the perifusion chamber and bubble trap (Fig. 4) following disassembly. Illustrated are the bubble trap (A), chamber caps (B and J), silicon gaskets (C and I), filter support discs (D and H), filters (E and G), chamber barrel with mounting bracket (F) and two-way stopcock for the injection of cells (K). Note the presence of a 8 cm ruler for scale.

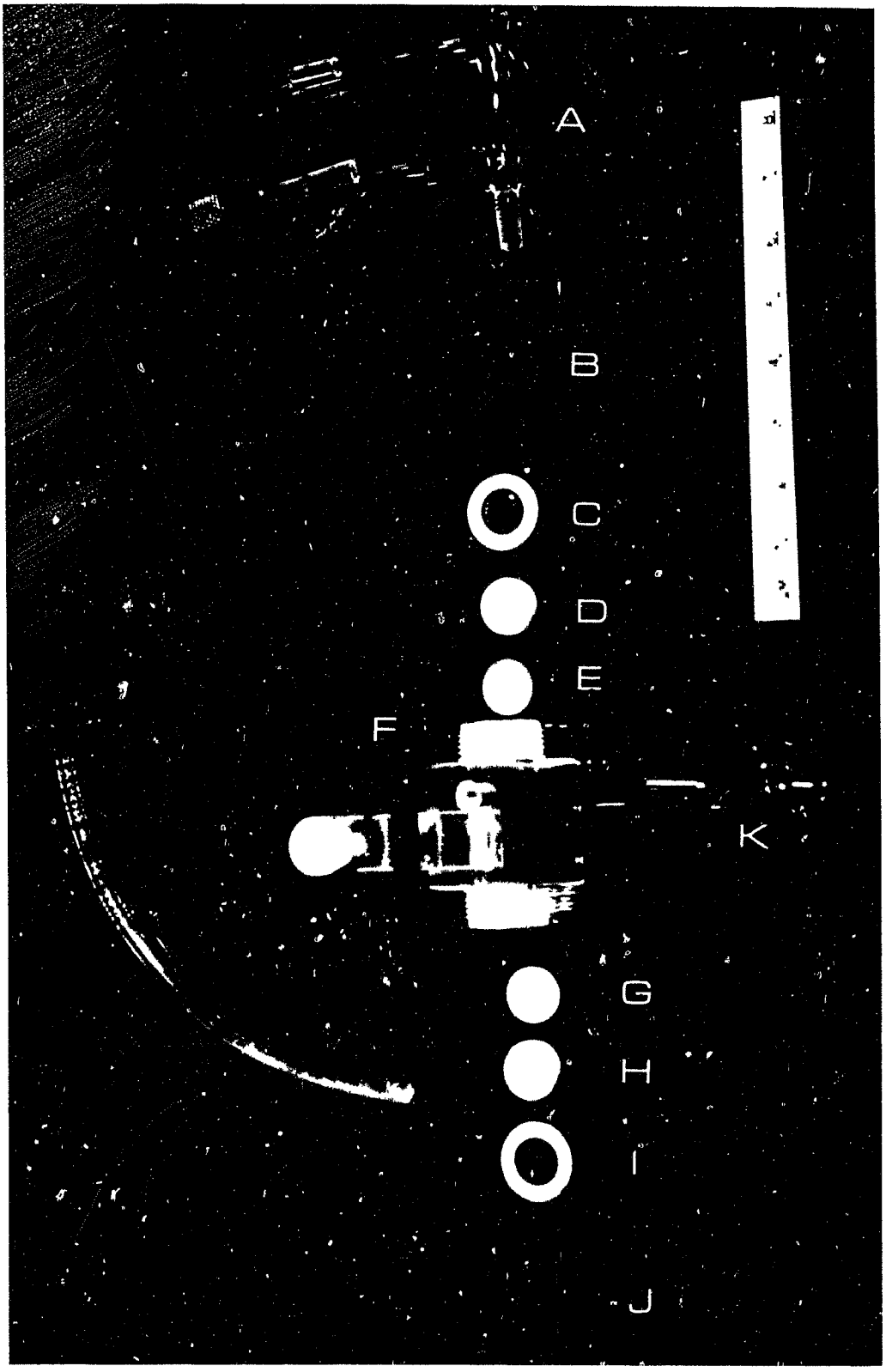


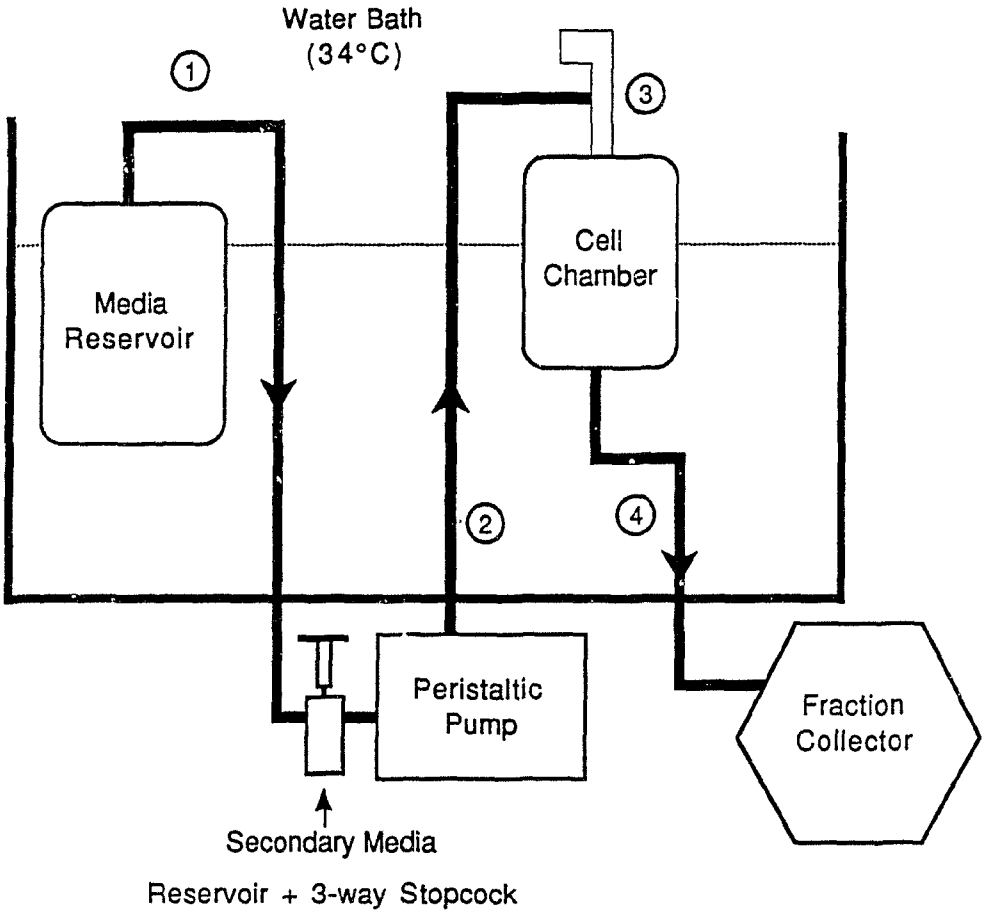
Figure 5.

interior of the chamber.

2. The perifusion system

Figure 6 is a schematic representation of one of the six perifusion systems utilized in parallel in these studies. Each apparatus consisted of a media reservoir, a reservoir lead connected via a three-way stopcock to silicon pump tubing within a peristaltic pump, an afferent lead, a bubble trap, the perifusion chamber and an efferent lead. The distal end of the reservoir lead (95 cm of Tygon tubing, 2.1 mm I.D.) was weighted and immersed in a media reservoir containing TC-199. A screw-type valve on this lead allowed for interruption of access to the media reservoir. The proximal end of this lead was connected via a three-way stop cock to silicon pump tubing (15 cm, 2.1 mm I.D.) which passed through a peristaltic pump (Model P-1, Pharmacia Fine Chemicals, Sweden). Inserted into the top of the three-way stopcock was a 5 ml plastic syringe barrel which acted as an secondary media reservoir. By stopping the pump, closing off the reservoir lead, opening the stopcock to the syringe reservoir and re-starting the pump, it was possible to transiently perifuse the contents of the perifusion chamber with treated media contained within the syringe barrel. The process was simply reversed to re-establish access with the primary media reservoir.

Fig. 6. A schematic representation of the perifusion system. Perifusion media was pumped from the media reservoir through the reservoir lead (1) which was connected to the peristaltic pump via a three-way stop-cock and associated secondary media reservoir. From the pump, the media flowed through the afferent lead (2) which was connected to the cell chamber by a bubble trap (3). The perifusate passed out the bottom of the chamber, through the efferent lead (4) where upon it was collected by a fraction collector. The media reservoir, afferent lead and cell chamber were maintained at 34 °C by a water bath. Arrow heads represent flow direction.



(1) Reservoir Lead
(2) Afferent Lead
(3) Bubble Trap
(4) Efferent Lead

Figure 6.

The pump tubing was connected by a ¼" quick-release nipple and coupling to the afferent lead (65 cm Intramedic polyethylene tubing, 1.19 mm I.D.) which in turn inserted into a glass bubble trap (Fig 5A). The bubble trap, which was inserted into the inlet of the chamber cap on the top of the perifusion chamber, minimizing the interruption of perifusate flow due to gas bubbles in the perifusion media. The media was pumped through the perifusion chamber, out the bottom chamber cap and through the efferent lead (95 cm, 1.19 mm I.D.) which was connected to a six-channeled fraction collector (Endotronics, Pioneer Cellular Engineering, Coon Rapids, MN). The media reservoir(s), perifusion chambers and the majority of afferent lead were immersed in a temperature controlled (34°C) water bath (Precision Scientific Co., Chicago, IL).

3. Cell perifusion

The day before a perifusion experiment, six aliquots of Bio-gel P2 beads (200-400 mesh size, 400 mg/aliquot) were put into separate glass incubation tubes (12 X 75 mm). The Bio-gel was then presoaked at 4°C overnight in 2 ml of TC-199 which contained 1% BSA to minimize the potential for nonspecific binding of chemical mediators and steroids to the carrier matrix (McNatty et al., 1984). Soaking overnight caused the beads to swell, approximately doubling the volume

of the gel-like material. On the day of the experiment, the supernatent was aspirated off and the Bio-gel which was resuspended in regular (0.1% BSA) TC-199.

While allowing the beads to resediment, each of the six perifusion systems were charged with warmed (34°C) TC-199 which had been gassed (95% O_2/5% CO_2) for 15 min. The perifusion media was supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin to minimize the potential for bacterial growth during the course of the perifusion. The supernatant was again aspirated off the Bio-gel which was then gently mixed with purified mouse Leydig cells ($1X10^6$ Leydig cells/500 μL TC-199/aliquot of Biogel). The Bio-gel/cell slurry was then carefully aspirated with pasteur pipettes, transferred into six separate 5 ml plastic syringes and immediately injected into each of the six cell perifusion chambers by way of the injection port on each chamber. The Biogel served two purposes: physical support of the cells and reduction of the chamber volume. By acting as a support matrix, the Biogel minimzed the progressive movement of cells which eventually resulted in some cell adhesion to the bottom 5 μM filter in the chamber. Reduction of the interchamber dead space in the system was advantageous in reducing the rate of catabolite accumulation, minimizing problems of treatment dilution and facilitating a rapid cell response to chemical mediators.

The chambers were then immersed into the water bath

up to the point where the glass bubble trap was inserted into the top chamber cap. The afferent lead just proximal to the connection with the bubble trap was also immersed as were the media reservoir(s), ensuring that the media flowing from the pump to the chamber was at the appropriate temperature (34°C). The cells in each of the chambers were then perifused with TC-199 by positive pressure at a rate of 12 ml/h using a separate peristaltic pump for each chamber. The perifusate from each chamber was collected at 10 min. intervals for approximately 5 hours using a fraction collector which can collect fractions from the six separate perifusion systems. Flow rate was periodically assessed through the course of the experiment by weighing the perifusate collected in plastic incubation tubes. By taring the weight of an empty tube, it was possible to monitor, and alter if necessary, the perifusion rate to insure a flow rate of 2 ml (2 g) of media/10 min.

The efluent during the first 50 min of each perifusion experiment was not utilized for experimental purposes to allow androgen production to stabilize. At this point (time = 0 min), collection of perifusate samples commenced. In most experiments, the cells were subsequently infused with TC-199 alone or TC-199 containing chemical mediators for 20 min, beginning 50 min into the experimental period. Cells were infused with the CaM antagonist, W7 (5-100 μM), its dechlorinated analogue, W5 (5-100 μM) or the

enzymatic inhibitors, mepacrine (20 μM) or nordihydroguaiaretic acid (NDGA; 50 μM) as indicated in the Results section. In certain experiments, cells were perifused with a combination of 100 μM W7 and 50 μM NDGA. In all experiments, one chamber acted as a control in that cells were treated in the same manner but the media contained no chemical mediators. There was a lag time of 30 min following the onset of the 20 min treatment infusion before any chemical mediators or steroidogenic response to those mediators were measureable in the collected efluent (Figs. 17B and 18). Therefore, it was possible to utilize the fractions collected over the first 70 min of the experimental period to establish the baseline androgen production. The degree of treatment dilution during this 20 min infusion was addressed in Study III (Fig. 20B) in the Results section.

In certain experiments, the cells were perifused with an additional 5 min pulse of 100 ng/ml LH beginning 45 min following the end of the initial 20 min treatment infusions (115-120 min after the beginning of the experimental period). The timing of LH treatment was important to insure that the maximal LH response occured coincidently with the maximal stimulatory response to 100 μM W7 (at $\approx$160 min). In other experiments, certain chambers were perifused for the entire experiment with media containing MIX (0.1 mM), 25-hydroxycholesterol (1.0 μM), mepacrine (20 μM) or NDGA (10

μM). Collected fractions were subsequently frozen at -20°C until RIA for androgens, cAMP or cGMP.

G. Radioimmunoassays

1. Androgens

Androgen production was measured by RIA of unextracted samples as described by Anakwe and Moger (1986). An antiserum (antibody B-8) raised against testosterone was generously provided by Dr. D.T. Armstrong (University of Western Ontario, London, Ont.). Due to crossreactivity with with other 17β-hydroxy-androgens as assessed by Dr Armstrong, all results refer to total androgen production. The percentage cross-reactions are as follows: testosterone 100%; 5α-dihydrotestosterone 98%; 5α-androstane-3α,17β-diol 58%; 5α-androstane-3β,17β-diol 24%; androstenedione and androsterone <2%.

Androgen assays were done in duplicate using 0.1 M phosphate steroid buffer (as described in Section II. C.). The testosterone standard was made mixing 50 μl of testosterone stock (100 ng/ml) into 5 ml of steroid buffer. Glass tubes (12 X 75 mm) were numbered in duplicate to correspond to each incubation or perifusion sample. For the analysis of incubation studies, 10 μl/tube of sample was used while perifusions required 50 μl/tube. 800 μl of

steroid buffer was added to the tube used to determine total counts while 600 μl was added to the tube utilized to ascertain non-specific binding (NSB). The standard curve consisted of tubes containing 0 (BO), 10, 25, 50, 100, 200, 300, 400 or 500 μl of testosterone standard (1 pg testosterone/μl). The appropriate volume of steroid buffer was then added to all tubes (standards and samples) to bring the total volume up to 500 μl/tube. A sufficient volume of radiolabelled testosterone was made up ($\approx$14,000 CPM/100 μl/tube) by air drying the appropriate volume of [^{3}H]testosterone stock and redissolving in steroid buffer (100 μl stock/10 ml steroid buffer). A 100 μl aliquot of this solution was then added to every tube. Lyophilized testosterone antibody had previously been dissolved in 0.9% saline to a concentration of 0.67% antibody and stored as 100 μl aliquots at -70°C until use. One such aliquot was thawed and diluted into 26 ml of steroid buffer. A 100 μl aliquot of this antibody solution was added to every tube except the total counts and NSB tubes. All tubes were then vortexed, covered and incubated overnight at 4°C.

The following day, steroid buffer was used to make up a suspension of dextran-coated charcoal (0.625% activated charcoal and 0.0625% dextran T-70) which was placed in ice and mixed with a magnetic stirrer for 15 min. A repeating pipettor was used to add 200 μl of this cold charcoal solution to each tube, with the exception of the total

counts tube. The tubes were then vortexed and incubated on ice for 10 min prior to centrifugation at 1000 X g for 15 min in a refrigerated centrifuge (4°C). The supernatent was then decanted into 7 ml scintillation vials to which 5 ml of scintillation cocktail (Formula-963 aqueous counting cocktail, NEN Research Products, Boston, Mass.) was added. The tubes were then briefly mixed and placed into the scintillation counter (1215 Rackbeta II Liquid Scintillation Counter, Wallac Oy, Finland) to determine the ammount of [^{3}H]testosterone which has bound to the antibody (ie. that which is not stripped from solution by the dextran-coated charcoal).

Counts per minute from the standard curve were transformed into logit (Y) = log (100/(100-% bnd Y)) where % bnd Y = ((CPM Y-NSB/BO CPM - NSB)) X 100. The logit versus log [standard] was plotted by the RIAPC RIA Calculation program (D. Rieger, 1988) and used to extrapolate the androgen concentrations in the samples. The assay sensitivity (90% BO) ranged from 5 to 8 ng/ml while specific binding (BO/total counts X 100) was consistently around 40%.

2. Cyclic nucleotides

Accumulation of cAMP and cGMP in cell incubation or perifusion samples was assessed using RIA kits and protocols

purchased from Biomedical Technologies Inc. (Stoughton, MA.). These kits employed a preconjugated double antibody separation system in an acetate buffer and could measure cyclic nucleotides in a range of 0.05-200 pmol/ml for both cGMP and cAMP. The BSA was removed from the samples prior to RIA by immersion in boiling water for 20 min followed by centrifugation at 1000 X g for another 20 min. The supernatent was then decanted and aliquoted (10-100 μL of sample/glass 12 X 75 mm assay tube). Total counts and NSB tubes as well as assay standards (0, 0.05, 0.1, 0.5, 2, 5 and 10 pmol/ml) were set up in duplicate. To increase assay sensitivity, the samples and standards were acetylated by adding 5 μL of 1 part acetic anhydride, 2 parts triethylamine. A 100 μl aliquot of the working tracer solution was then added to all tubes (^{125}I-succinyl cAMP- or cGMP-tyrosine methyl ester diluted with sodium acetate buffer, pH 6.2, containing normal rabbit IgG, phosphodiesterase inhibitors and sodium azide). The working antibody solution containing specific cGMP or cAMP antiserum and sheep anti-rabbit IgG was then added to all tubes with the exception of the total counts and NSB tubes. To assess non-specific binding, the NSB tubes recieved 100 μl of NSB reagent which was identical to the working antibody solution but without the specific antiserum while the total count tube recieved only the radiolabelled cyclic nucleotide. The tubes were then gently shaken, covered and incubated at 4° C

for 18-20 hours. At the end of the incubation period, 1 ml of cold acetate buffer was added to all tubes, except the total count tubes, and they were centrifuged at 1000 X g for 20 min. Following centrifugation, the supernatants were separated from the pellets by decantation and the tubes counted for 1 min in a gamma particle counter (LBK-Wallac Clinigamama 1272, Finland).

Counts per minute from the cAMP/cGMP standard curve were transformed, plotted and used to extrapolate sample values using the same methods as described for the androgen assays.

H. Data and statistical analysis

1. Synergism ratio

In experiments where synergistic interactions between agents were investigated, data were expressed, in most cases, as a synergism ratio (SR) such that SR = (XY)-C / (X-C)+(Y-C) wherein XY is the steroidogenic response upon coexposure to a certain combination of two agents, C represents the basal androgen production in the absence of any stimulatory agent, while X and Y represent the response to each of the two agents individually. Therefore, SR>1 ($p \leq 0.05$) was indicative of a synergistic response while a SR=1 represented an additive response.

2. Statistical analysis

Statistical significance of SR was assessed using a single mean Student's t-test with $p \leq 0.05$ considered to be statistically significant. Other data were assessed for statistical significance using an unpaired two-tailed Student's t-test or analysis of variance (ANOVA) with $p \leq 0.05$ considered to be statistically significant. A Scheffe F-test was utilized to assess the dose responsiveness of a treatment. The EC_{50} values in certain experiments were calculated using the Grafpad/Inplot (Version 3.0) computer graphing package. Mean values of results are shown with the standard error of that mean as an estimate of variance.

III. STUDY I

Type 1 and type 2 isoenzymes of cAMP-dependent protein kinase in Leydig cell steroidogenic function

A. Introduction

It has been well established that luteinizing hormone (LH) from the anterior pituitary acts as the predominant stimulatory mediator of steroidogenic activity in the Leydig cell, acting through the cyclic adenosine 3',5'-monophosphate (cAMP) second messenger system (Mendelson et al., 1975; Cooke et al., 1976). Some studies have suggested that the steroidogenic apparatus can be activated by LH in a cAMP-independent manner. A detectable increase in androgen production has been noted prior to any discernable elevation in intracellular cAMP levels following exposure of both freshly isolated and tumour Leydig cells to gonadotropins (Mendelson et al., 1975; Peirera et al., 1987). However, a study by Peirera and coworkers (1987) strongly suggested that cAMP was still involved at some level (Peirera et al., 1987). They incubated Leydig tumour cells with the R_p diastereoisomer of cyclic adenosine 3',5'-phosphorothioate (R_p-cAMP), which binds to but does not activate PK-A (Eckstein, 1985). Acting as a competitive cAMP antagonist, R_p-cAMP attenuated the steroidogenic response to levels of LH which did not generate any measurable increase in cAMP

levels (Peirera et al., 1987). This same study showed that both the type 1 (T1) and type 2 (T2) isoenzymes of cAMP-dependent protein kinase (PK-A) could be isolated from the mouse Leydig tumour cells. This reaffirmed previous studies by Cooke and coworkers (1976) in which both of the predominant isoforms of PK-A were isolated from normal rat Leydig cells. Based on the relative phosphotransferase activities of the PK-A isoenzymes, Peirera et al. (1987) concluded that the T2 isoform was not of particular functional importance in the Leydig tumour cell. The purpose of this current study was to more fully characterize the functional role of these two major isoenzymes of PK-A in the control of steroidogenic mechanisms. To do so, cAMP analogues were utilized which are selective for either of the two cAMP binding sites (S1 and S2) on the regulatory subunits of the T1 or T2 PK-A isoenzymes. Coexposure of the mouse Leydig cells to aminohexylamino-cAMP (AHA), a S1/T1-selective analogue and N^6-benzoyl-cAMP (B) an analogue selective for S2 of either T1 or T2 isoenzyme should elicit a synergistic increase in androgen production if type 1 PK-A is present and functional in the cell. Alternatively, the presence of the T2 isoenzyme would become evident should a nonadditive stimulation of steroidogenesis result upon combined exposure to a S1/T2 analogue, 8-thiomethyl-cAMP (TM), and the site 2, type-nonselective analogue (B). Using these cAMP analogues in combination or coincubated with LH,

cAMP or forskolin, we have ascertained that both PK-A isoenzymes are functionally important in the control of murine Leydig cell steroidogenesis.

B. Results

1. The effect of site/type-selective analogues of cAMP on androgen production

To ensure optimum site/type selectivity, it is necessary to utilize concentrations of the site/type-selective cAMP analogues which minimally but consistently stimulate steroidogenesis (Beebe et al., 1988). A dose response study revealed that a lower concentration of AHA (1μM) was sufficient than was the case for B and TM (10 μM) (Fig 7). Higher concentrations of AHA ($\geq$30 μM) or B (100 μM) inhibited androgen production, possibly due to PK-A activation of cyclic nucleotide PDE (Gettys et al., 1987).

After the appropriate treatment concentrations for the cAMP analogues was determined, Leydig cells were incubated with AHA (1uM; S1,T1), TM (10uM; S1,T2) or B (10uM; S2,T1/2) alone or in combinations to selectively activate type 1 PK-A (AHA + B), type 2 PK-A (TM + B) or both isoenzymes (AHA + TM). All of these treatment paradigms stimulated androgen production (Table I) with the analogue combinations acting in a synergistic manner to increase

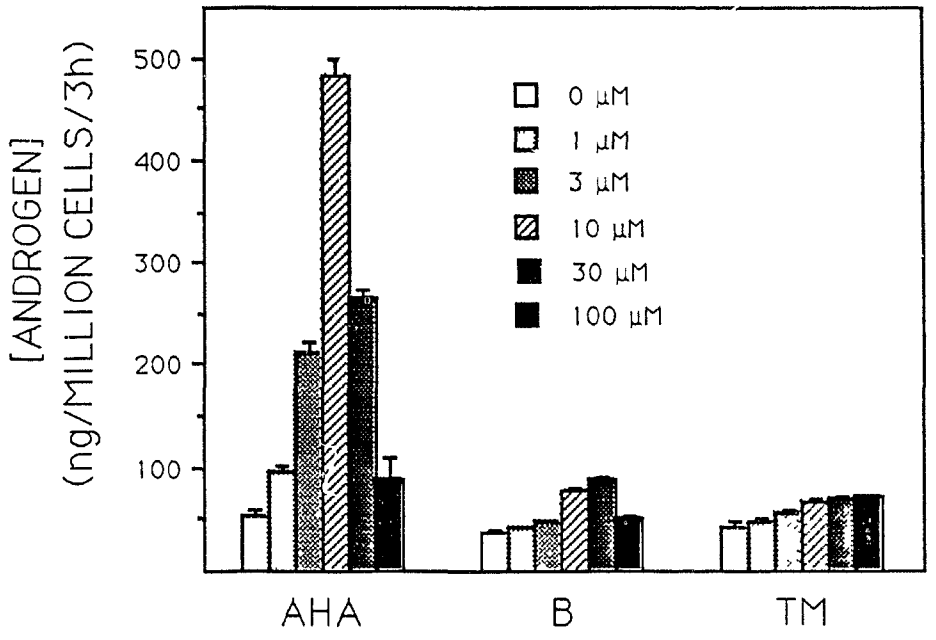


Figure 7. The effect of site/type-selective cAMP analogues on androgen production by mouse Leydig cell incubations. Increase in androgen production upon exposure of mouse Leydig cells to AHA (S1,T1), B (S2,T1/2) or TM (S1,T2) cAMP analogues. Data represents mean ± SEM of a representative experiment.

TABLE I. The effect of site/type-selective cAMP analogues, alone and in combination, on androgen production by mouse Leydig cells.

TREATMENT	[ANDROGEN] (ng/MILLION CELLS/3h)
CONTROL	68.8 ± 12.8
1 μM AHA-cAMP	115.2 ± 21.2
10 μM B-cAMP	118.5 ± 18.5
10 μM TM-cAMP	108.1 ± 18.3
AHA + B	562.8 ± 91.9
B + TM	404.8 ± 53.7
AHA + TM	352.1 ± 59.7

* Mean ± SEM of 13 separate experiments

steroidogenic output. Calculation of synergism ratios (SR; as described in the Materials and Methods) from this data revealed that activation of either isoenzyme of PK-A resulted in a significant synergistic increase in androgen production (SR $\geq$ 1, $p \leq 0.05$) with significantly greater activation occurring via the type 1 PK-A (Fig. 8, SR = 5.72 $\pm$ 0.46 SEM) than through the type 2 PK-A (SR = 4.22 $\pm$ 0.42) as determined by analysis of variance and a Scheffe F-test. Co-exposure to analogues which bind to S1 of each isoenzyme (AHA + TM) should not elicit a synergistic response. However, a significant degree of interaction was evident (SR = 2.88 $\pm$ 0.15, $p \leq 0.05$) albeit significantly smaller than that elicited with selective PK-A activation. This finding illustrates the difference between selectivity and specificity of the cAMP analogues. The structural alterations within the cyclic nucleotide derivatives increase their site/type-selectivity above that of endogenous cAMP without providing absolute specificity for a particular binding site or isoenzyme.

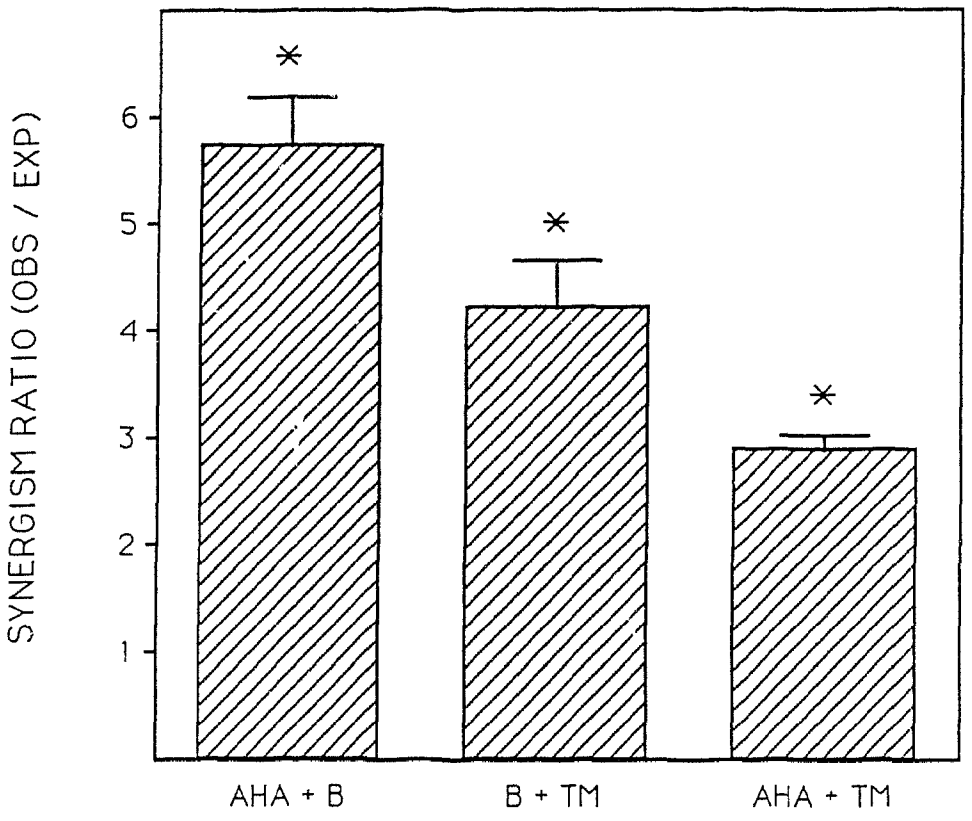


Figure 8. Synergism between site/type-selective cAMP analogues. Increase in androgen production upon coexposure of mouse Leydig cells to AHA (S1,T1) + B (S2,T1/2), TM (S1,T2) + B and AHA + TM. Note: y-axis in this figure is the synergism ratio (SR) calculated as described in Materials and Methods. * denotes significant statistical difference from additive response (SR = 1.0), $p \leq 0.05$ and a statistically significant treatment effect as determined by a Scheffe F-test. Data represents the mean ± SEM of 13 separate experiments.

2. The effect of phosphodiesterase inhibition on the steroidogenic response to the site/type selective cAMP analogues

In this experiment, we addressed the possibility that the site/type-selective analogues could be eliciting at least some of their stimulatory effects through the inhibition of cyclic nucleotide phosphodiesterase (PDE) activity. With this in mind, the steroidogenic effects of the analogues were assessed in the presence or absence of 0.1 mM isobutylmethylxanthine (MIX), a nonspecific PDE inhibitor (Bergstran et al., 1977). If the steroidogenic response to the cAMP analogues was mediated through sparing of cyclic nucleotides then chronic PDE inhibition should reduce the response. As illustrated in Fig. 9, PDE inhibition with MIX had no effect on the response to the cAMP analogues, suggesting that the sparing of cyclic nucleotides was not a factor in the response.

3. Interaction between cAMP and the site/type-selective cAMP analogues

Leydig cells were coincubated with each of the cAMP analogues in combination with exogenous cAMP (1mM) or endogenous cAMP generated through activation of adenylate cyclase with the diterpene, forskolin (0.5 μM). Both of

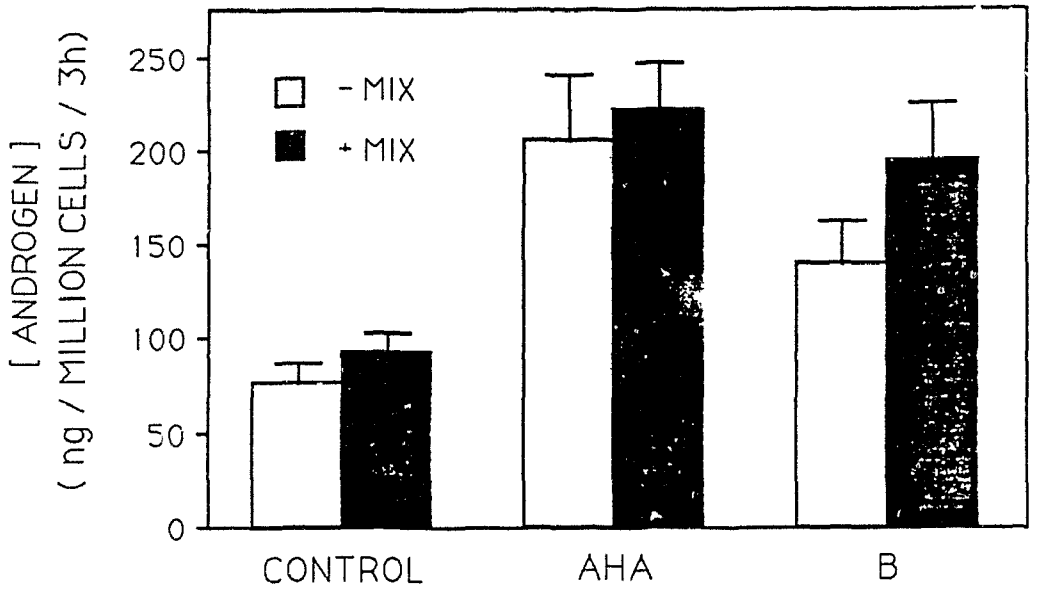


Figure 9. The effect of phosphodiesterase inhibition on the steroidogenic response to AHA and B. Increase in androgen production upon exposure of mouse Leydig cells to A) AHA (1 μM) or B) B (10 μM) ± 0.1 mM MIX. Data represents the mean ± SEM of 4 separate experiments.

these treatment paradigms were capable of eliciting a minimal cAMP-mediated increase in androgen production (control: 58.5 ± 11.6 vs forskolin: 74.55 ± 12.19 ng androgen/million cells /3h; control 63.28 ± 10.4 vs cAMP: 77.1 ± 12.5 ng androgen/million cells /3h). Figure 10 illustrates that a synergistic response was evident with the cells co-exposed to exogenous cAMP, predominantly through type 1 PK-A though significant interaction via the type 2 isoenzyme was also evident (AHA SR = 2.56 ± 0.65, $p \leq 0.04$; B SR = 1.75 ± 0.22, $p \leq 0.012$; TM SR = 1.54 ± 0.24, $p \leq 0.05$). The addition of 0.5 μM forskolin also elicited a synergistic increase in androgen production (Fig. 10) albeit somewhat less than that noted with cAMP and predominantly through the type 1 isoform (AHA SR = 1.57 ± 0.2, $p \leq 0.025$; B SR = 1.50 ± 0.24, $p \leq 0.05$; TM SR = 1.12 ± 0.13, $p \leq 0.19$). The possibility that forskolin could be inhibiting cellular activation in a cAMP-independent fashion was investigated. In preliminary experiments, Leydig cells were incubated with LH (0.3-100 ng/ml) or the cAMP analogues and 1 μM 1,9-dideoxyforskolin (DDF), a naturally occurring analogue of forskolin which does not activate adenylate cyclase (Laurenza et al., 1989). As can be seen in Fig. 11, DDF significantly inhibited the steroidogenic response to LH in this experiment although the EC_{50} for the gonadotropin (3.8 ± 1.64 ng/ml LH) was unaffected (4.23 ± 1.55 ng/ml). The steroidogenic response to the cAMP analogues (AHA 89.5 ±

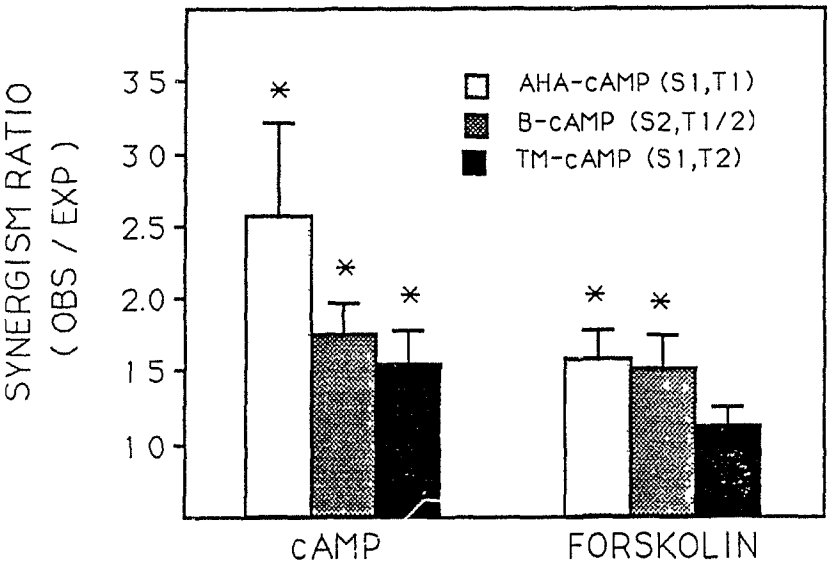


Figure 10. Synergism between site/type-selective cAMP analogues and exogenous cAMP or forskolin. Synergistic increase in androgen production upon coexposure of mouse Leydig cells to AHA (S1,T1), B (S2,T1/2) or TM (S1,T2) and 1.0 mM cAMP or 0.5 μM forskolin. * denotes significant statistical difference from additive response (SR = 1.0), $p \leq 0.05$. Data represents the mean ± SEM of 4 separate experiments.

2.75 ng androgen/million cells/3h; B - 99.0 ± 5.9; TM-69.0 ± 5.1; AHA + B - 292 ± 13.9; TM + B - 194 ± 3.7) was also inhibited by DDF in this experiment, though not significantly (AHA/DDF - 74.5 ± 4.6; B/DDF - 73.5 ± 4.5; TM/DDF - 61.5 ± 3.3; AHA + B - 263 ± 30; TM + B - 189 ± 10.4).

4. Interaction between LH and site/type-selective cAMP analogues

The Leydig cells were incubated with luteinizing hormone (LH) (0.3 - 100 ng/ml) alone, or in combination with each of the cAMP analogues. In this case, a significant synergistic interaction is evident with low levels of LH via both protein kinase isoenzymes (Fig. 12), with interaction through type 1 PK-A predominating (0.3 ng/ml LH: AHA SR = 2.40 ± 0.6, $p \leq 0.05$; B SR = 1.75 ± 0.2, $p \leq 0.02$; TM SR = 1.51 ± 0.15, $p \leq 0.025$; 1.0 ng/ml LH: AHA SR = 1.86 ± 0.27, $p \leq 0.025$; B = 1.70 ± 0.22, $p \leq 0.025$; TM = 1.58 ± 0.31, $p \leq 0.73$).

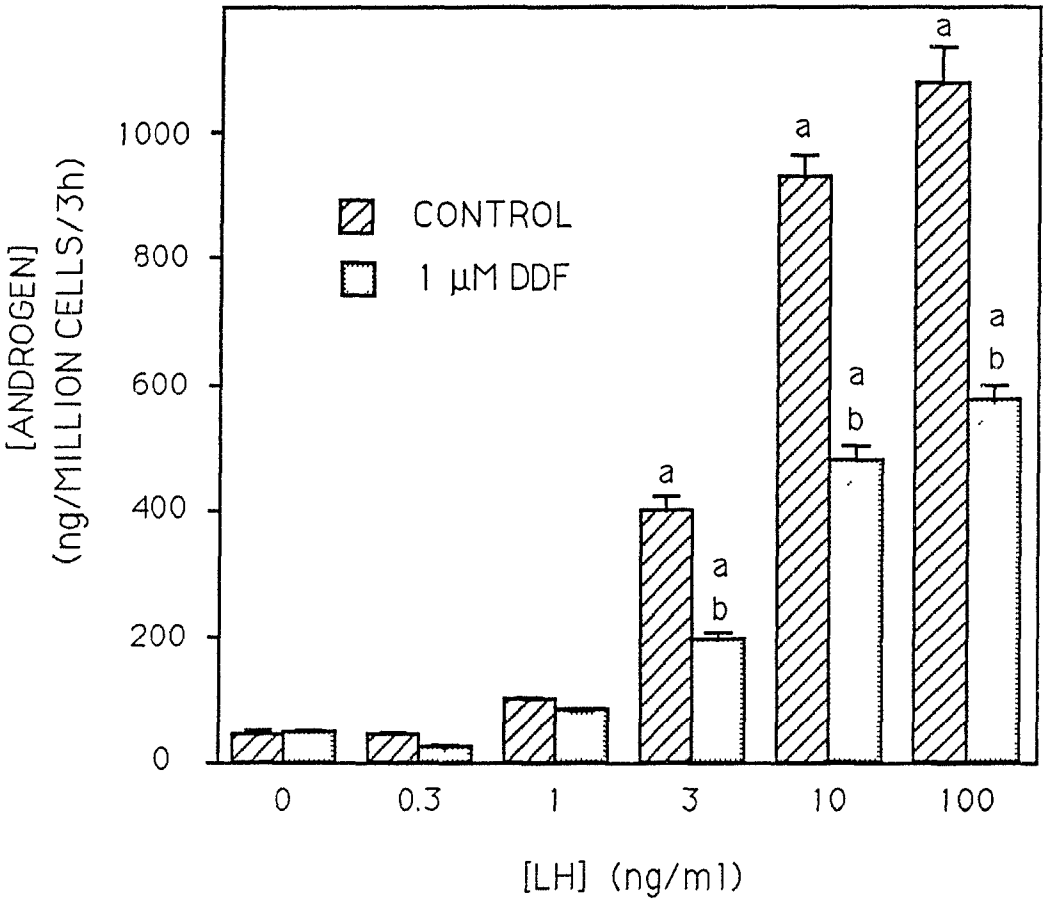


Figure 11. The effect of 1,9-dideoxyforskolin on LH-stimulated androgen production in Leydig cell incubations. Cells were incubated with LH (0-100 ng/ml) in the presence or absence of 1 μM 1,9-dideoxyforskolin (DDF). "a" denotes a statistical difference from control production while "b" indicates a significant treatment effect of DDF as calculated by a Scheffe F-test. Data represents the mean ± SEM of a representative experiment.

Figure 12. The steroidogenic response of Leydig cells to LH, alone or in combination witn site/type-selective cAMP analogues. A) Androgen production in response to LH (0-100 ng/ml). B) Synergistic increase in androgen production upon coexposure of mouse Leydig cells to AHA (S1,T1), B (S2,T1/2) or TM (S1,T2) and LH (0.3-100 ng/ml). * denotes significant statistical difference from additive response (SR = 1.0), $p \leq 0.05$. Data represents the mean ± SEM of 4 separate experiments.

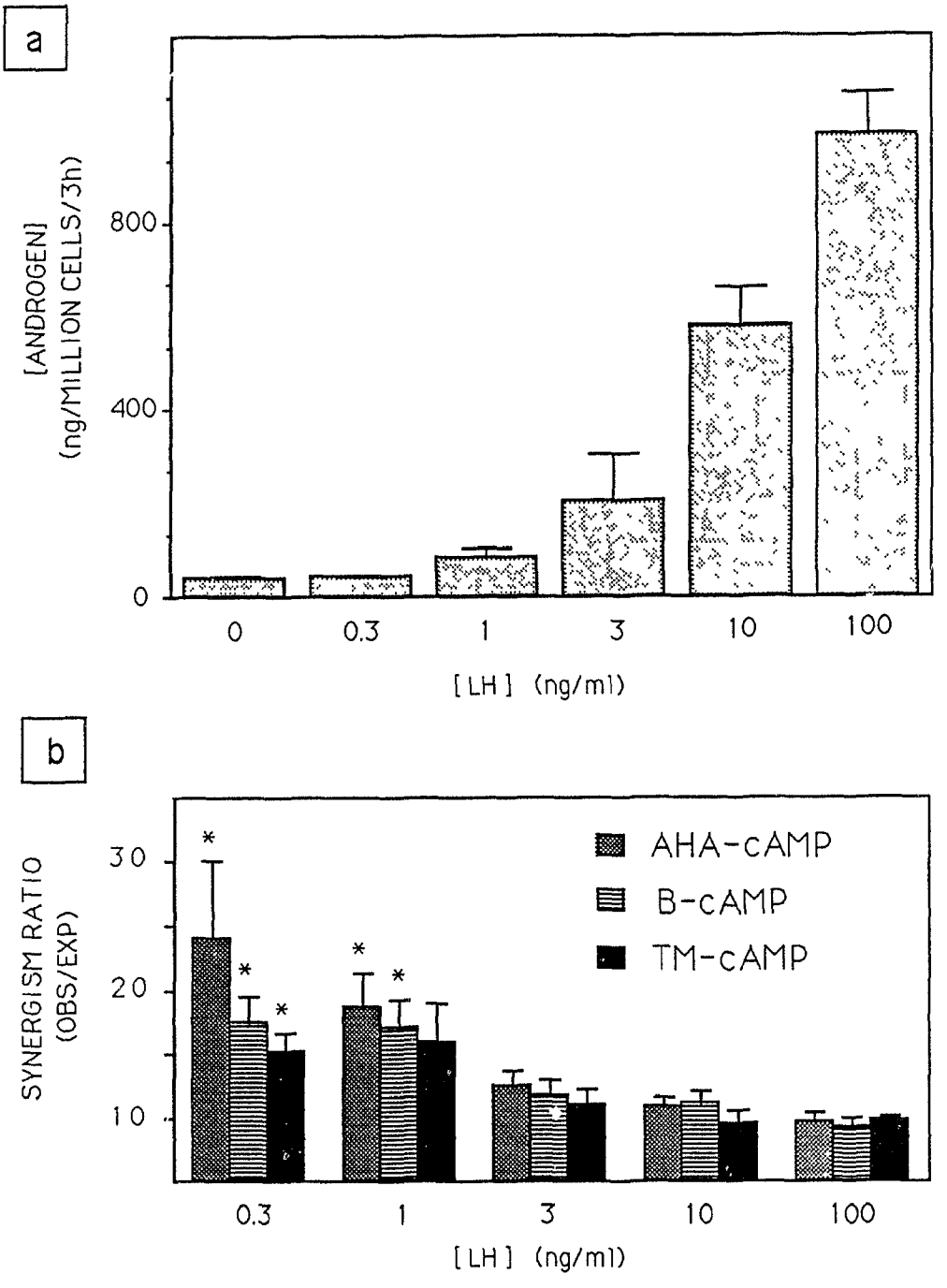

a
[ANDROGEN]
(ng/MILLION CELLS/3h)
800
400
0
0
0.3
1
3
10
100
[LH] (ng/ml)
b
SYNERGISM RATIO
(OBS/EXP)
30
20
10
AHA-cAMP
B-cAMP
TM-cAMP
*
0.3
1
3
10
100
[LH] (ng/ml)

Figure 12.

C. Discussion

The development of cAMP analogues selective for the binding sites on the regulatory subunits of two isoenzymes of cAMP-dependent protein kinase has allowed for a more thorough examination of the next step in the cAMP cascade. While Cooke et al., (1976) originally isolated both type 1 and 2 PK-A from cultured rat Leydig cells, Peirera and coworkers (1987) were able to assess the contribution of each of the isoenzymes in the phosphorylation of target proteins. They could isolate both type 1 and 2 PK-A from MA-10 Leydig tumour cells but found that the type 2 isoenzyme was responsible for only a minor percentage of the total detectable phosphotransferase activity (15-20%). It is difficult, if not impossible, to characterize the functional importance of the T2 isoform in the Leydig cell from this quantitative measurement of enzyme activity (Peirera et al., 1987). The use of the MA-10 Leydig tumour cell line in that study further complicates the extrapolation of these findings to the normal cell type. The MA-10 tumour cells have functional characteristics which differ from the normal Leydig cells including the inability to synthesize testosterone (Ascoli and Puett, 1978; Ascoli, 1981).

Indeed, our findings suggest that the type 2 isoenzyme does have an important physiological role in the mouse Leydig cell exceeding that which might be construed

from the phosphotransferase activity measured in the mouse tumour cell (Peirera et al., 1987). Coexposure to analogues selective for the two nucleotide binding sites on the type 2 regulatory units (TM and B) consistently resulted in significant synergistic increase in androgen production albeit significantly reduced from that noted with type 1 activation (AHA and B; Table I, Fig. 8). This would indicate that both PK-A isoenzymes were present and functionally active in the mouse Leydig cell. This position was supported by the synergistic interaction via both type 1 and 2 isoenzymes resulting upon coexposure of the cells with exogenous cAMP (Fig. 10) or low stimulatory levels of LH (Fig. 12) and the cAMP analogues.

The significant difference in the relative capabilities of the site/type-selective analogue pairs (ie. AHA + B vs TM + B) in stimulating steroidogenesis could relate to a number of different factors. It could, in part, be a manifestation of a lower phosphotransferase activity of the T2 PK-A isoform as noted in the Leydig tumour cells (Pereira et al., 1987). If this is the case, it would imply that the two isoenzymes perform identical regulatory functions within the cell and this would seem unlikely.

The phosphorylation state of the type 2 isoform may also influence its activition by the cAMP analogues. It has been shown in other cell types that type 2 PK-A exists primarily in a phosphorylated and therefore, hypersensitive

state (Rangel-Aldao et al., 1979). If this was the case in the Leydig cell, a reduced response to the T2-selective cAMP analogues could reflect a relative absence of intact holoenzyme available for cAMP binding. This heightened affinity for cAMP arising from autophosphorylation of the type 2 isoform could facilitate its activation by basal levels of cAMP facilitating the synthesis of androgens in the absence of steroidogenic activators.

The lack of synergism noted via both PK-A enzymes at higher but submaximal concentrations of LH was not surprising. The cAMP analogues were competing with endogenously generated cyclic nucleotides for the binding sites on the regulatory subunits of the kinase isoenzymes. As the synthesis of cAMP increased with greater LH stimulation of adenylate cyclase (Mendelson et al., 1975; Cooke et al., 1976), the selective effect of the analogues was lessened as the competition for binding sites escalated (Corbin et al., 1988). By this same reasoning, to elicit the maximal degree of synergism, the site/type-selective cAMP analogues should be utilized at concentrations which consistently but minimally stimulate androgen production (Fig. 7; Table I) (Beebe et al., 1988).

It is interesting to note that the magnitude of interaction between cAMP and the PK-A isoenzymes seemingly varied with the means by which the cyclic nucleotides were introduced into the cell. Synergistic interaction with the

analogues was more prevalent with cAMP provided exogenously (Fig 10) or generated endogenously with LH (Fig. 12) than that achieved upon coexposure to forskolin (Fig. 10). Although synergism with the S1,T2 analogue, TM, was virtually abolished, quantitatively, there was a greater loss of synergism attained with the S1,T1 analogue, AHA (Fig. 10). The lack of synergism with forskolin relative to that noted with LH/cAMP is difficult to explain as all three treatment paradigms seemingly stimulate steroidogenesis via the same cAMP messenger cascade. However, the interaction of forskolin with adenylate cyclase (Seamon et al., 1981) represents only one of the functional activities of the diterpene. Recently, it has become apparent that forskolin has a number of functions independent of adenylate cyclase activation (Laurenza et al., 1989) including the inhibition of glucose transport (Joost et al., 1988; Amrolia et al., 1988) and modulation of ion channels (Coombs and Thompson, 1987; Wagoner and Pollatta, 1988; Yanagibashi et al., 1989). Exposure of the Leydig cell to forskolin could incite cellular event(s) independent of cAMP generation, events which may not occur via LH stimulation or with exogenous cAMP. In doing so, forskolin may induce changes in the intracellular environment which are not optimum for cooperative activation of the cAMP-dependent kinases in general. Indeed, even though the generation and binding of cAMP to the regulatory subunit were comparable, exposure to

1 μM forskolin was less effective than human chorionic gonadotropin (hCG) in stimulating steroidogenesis (Dufau et al., 1987). Much lower levels of forskolin (pM) actually inhibited both cAMP generation and androgen production in the rat Leydig cell (Dufau et al., 1987) while higher forskolin concentrations ($\geq$ 3.12 μM) inhibited the transport of 2-deoxy-D-glucose into the cell (Amrolia et al., 1988). These effects could explain the inability of maximally stimulatory levels of forskolin to increase androgen production to the same extent as LH in the mouse Leydig cell (Moger and Anakwe, 1983).

One criterion for establishing cAMP-independent actions of forskolin is the ability of its inactive analogue, DDF, to reproduce a forskolin-like effect (Laurenza et al., 1989). Preliminary findings using DDF (Section 3., Fig. 11) suggested that the concentration of forskolin utilized in this study (Fig. 10) may indeed have had inhibitory effects on cAMP-mediated activation of steroidogenesis underlying the activation of adenylate cyclase. Further investigation of cAMP-independent activities of forskolin in the murine Leydig cell is certainly warranted.

It is apparent that two major isoenzymes of cAMP-dependent protein kinase are present and active in the murine Leydig cell although the type 1 isoenzyme is functionally dominant in the stimulatory control of androgen

production by LH. It would appear that forskolin may have cAMP-independent activities which reduce the ability of the cyclic nucleotide to synergistically activate steroidogenesis through both PK-A isoenzymes.

IV. STUDY 2

Synergistic interaction between cyclic nucleotide second messenger systems in the mouse Leydig cell

A. Introduction

Though undoubtably important, LH and the cAMP second messenger system are not the only acute stimulatory mediators of steroidogenesis in the Leydig cell. Exposure to atrial natriuretic factor (ANF) has been shown to stimulate androgen production by both the mouse and rat Leydig cell (Bex and Corbin, 1985; Pandey et al., 1986b; Foresta et al., 1987). Indeed, ANF stimulated steroidogenesis in the mouse Leydig cell to a degree akin to that seen with maximally stimulatory levels of LH (Pandey et al., 1986b). The minimally effective concentration of ANF which stimulated the rat Leydig cell (10 pM; Foresta et al., 1987) may or may not be within the range of plasma ANF concentrations in the rat (Anderson and Bloom, 1986; Thibault et al., 1988). The physiological relevance of ANF-mediated stimulation of androgen production in the mouse is unknown as the concentration of ANF required to activate testicular steroidogenesis in the mouse (nM) were somewhat higher than blood-borne levels of the natriuretic peptide (Bex and Corbin, 1985; Mukhopadhyay et al., 1986a; Pandey et al., 1986a).

This ANF-mediated increase in androgen production did

not result from any increase in cAMP production (Mukhopadhyay et al., 1986a; Pandey et al., 1986b) but instead, was elicited through the increased synthesis of cGMP (Mukhopadhyay et al., 1986b). Interaction between the two cyclic nucleotide messenger cascades has been established in a number of different tissues (Goldberg et al., 1975). In many instances, "crosstalk" between the two systems was found to be adversarial in that the guanosine messenger system opposed cellular activities mediated through the cAMP cascade and vice versa (for review, see Goldberg et al., 1975). An example of this "Yin-Yang" relationship (Goldberg et al., 1975) was noted in cultured tumour Leydig cell in which ANF stimulated cGMP synthesis but reduced intracellular cAMP levels and attenuated the steroidogenic responsiveness to high stimulatory levels of LH (Pandey et al., 1985).

It is important to note that there has also been evidence in some tissues of a cooperative relationship between the two cyclic nucleotide systems wherein cAMP and cGMP acted synergistically (Nakamura and Gold, 1987; Ho et al., 1988; Furman and Tanaka, 1989). With this in mind, we decided to investigate the possibility of a cooperative relationship between LH/cAMP and ANF/cGMP in the murine Leydig cell such that the two hormonal mediators could be interacting to synergistically bolster androgen production. We coexposed the Leydig cells to ANF and LH or ANF/cGMP and

the site/type-selective cAMP analogues to see if a synergistic response would be elicited. Indeed, low concentrations of ANF and LH combined to synergistically stimulate steroidogenesis. Alternatively, exposure of the Leydig cells to either ANF or cGMP and these cAMP analogues elicited a synergistic rather than additive increase in androgen production, suggesting that ANF/cGMP may act cooperatively with LH/cAMP in the stimulatory control of androgen production in the mouse Leydig cell.

B. Results

1. Synergistic interaction between LH and ANF

As can be seen in Fig. 13, ANF stimulated androgen synthesis by mouse Leydig cells (EC_{50} = 1.51 ± 0.11 nM), as has been noted in a number of previous studies (Bex and Corbin, 1985; Pandey et al., 1986b, Mukhopadhyay et al., 1986a,b). This stimulation was accompanied by a consistent stimulation of cGMP accumulation even at low concentrations of ANF (Fig. 14A) with no significant stimulation of cAMP synthesis (Fig. 14B).

Incubation studies in which Leydig cells were co-exposed to 1 nM ANF and various concentrations of LH (Fig. 15) suggested that the two hormones could interact cooperatively to influence androgen production (Fig. 15A).

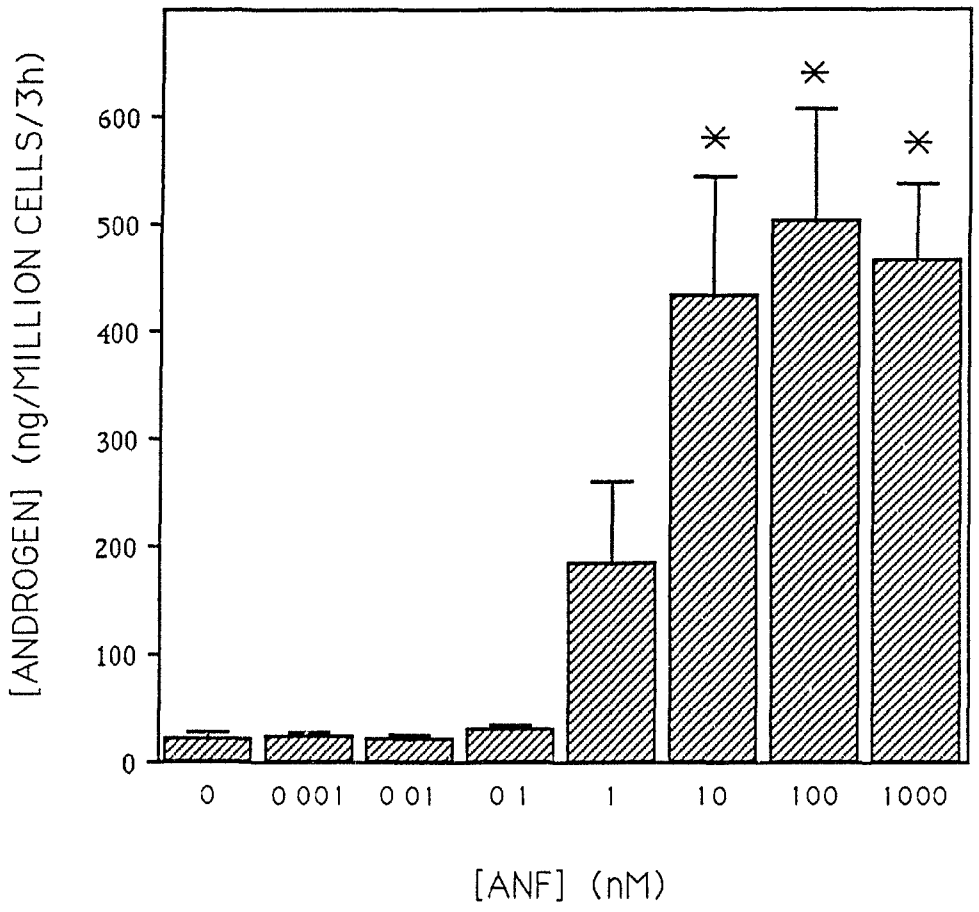


Figure 13. The effect of atrial natriuretic factor on androgen production in Leydig cell incubations. Cells were exposed to ANF (0 - 1000 nM) during 3 h static incubations as described in the **Materials and Methods** section. * denotes a statistically significant stimulation over control androgen production. All data represents means ± SEM of 6 separate experiments.

Figure 14. The effect of ANF on cyclic nucleotide production by Leydig cells in incubation. Cells were exposed to ANF (1 nM) during 3 h static incubations as described in the **Materials and Methods** section. The incubation media was subsequently radioimmunoassayed for cGMP and cAMP. The effect of ANF on the accumulation of cAMP (B) was not significant while the effect on cGMP was consistently stimulatory (A). All data represents means of duplicate determinations of 5 (Fig. 14A) or 13 (Fig. 14B) separate experiments.

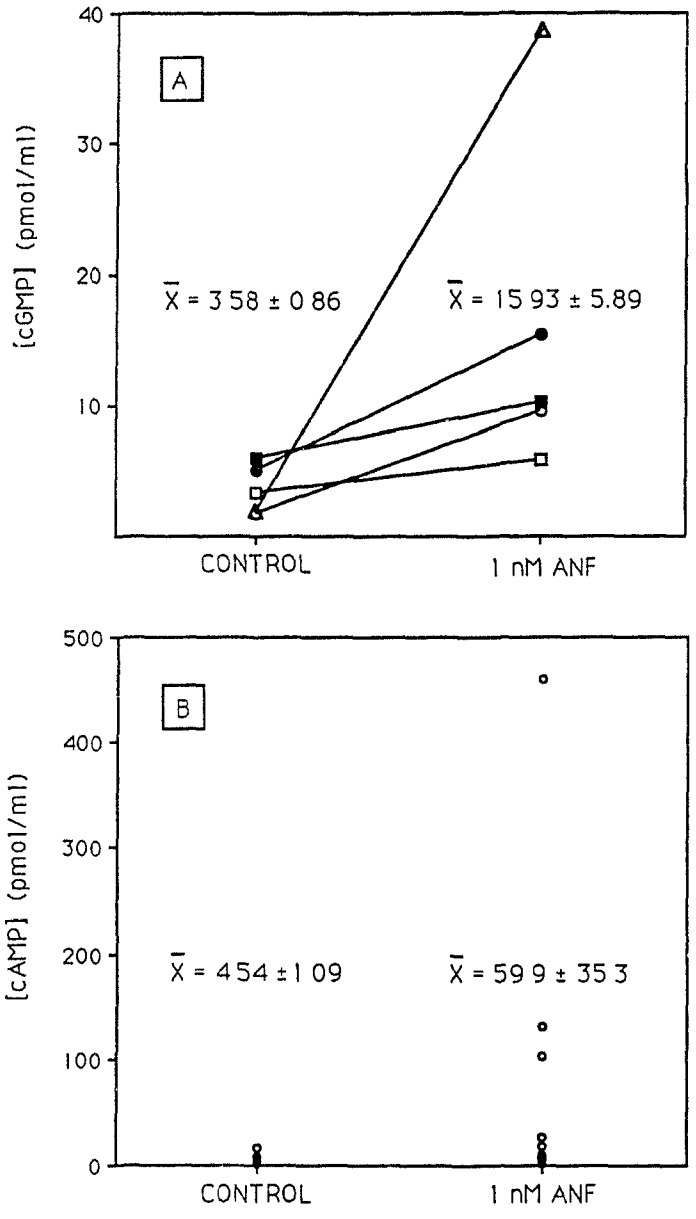
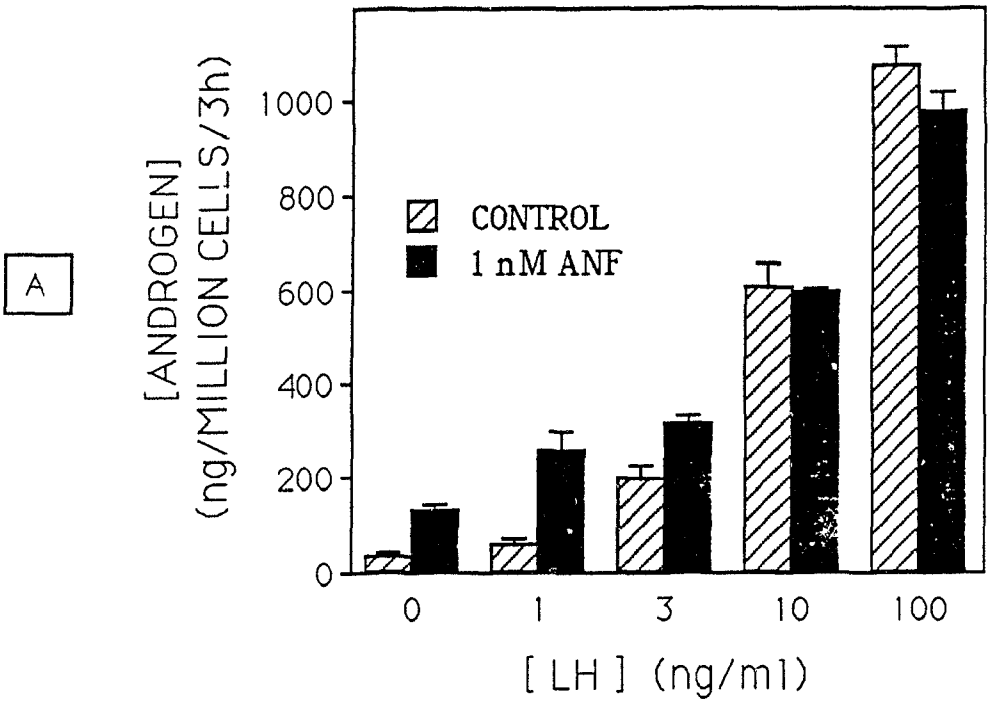

A
[cGMP] (pmol/ml)
40
30
20
10
X̄ = 3 58 ± 0 86
X̄ = 15 93 ± 5.89
CONTROL
1 nM ANF
B
[cAMP] (pmol/ml)
500
400
300
200
100
0
X̄ = 454 ± 1 09
X̄ = 59 9 ± 35 3
CONTROL
1 nM ANF

Figure 14

Figure 15. A synergistic interaction between LH and ANF. A) The effect of coexposure of mouse Leydig cells to ANF (1 nM) and LH (0.3-100 ng/ml) on androgen production. B) The synergistic interaction between ANF and LH on androgen production by mouse Leydig cell * denotes a statistically significant difference from an additive response (SR = 1.0), $p \leq 0.05$. Data represents the mean ± SEM of 3 separate experiments.


A
[ANDROGEN]
(ng/MILLION CELLS/3h)
1000
800
600
400
200
0
CONTROL
1 nM ANF
0
1
3
10
100
[LH] (ng/ml)

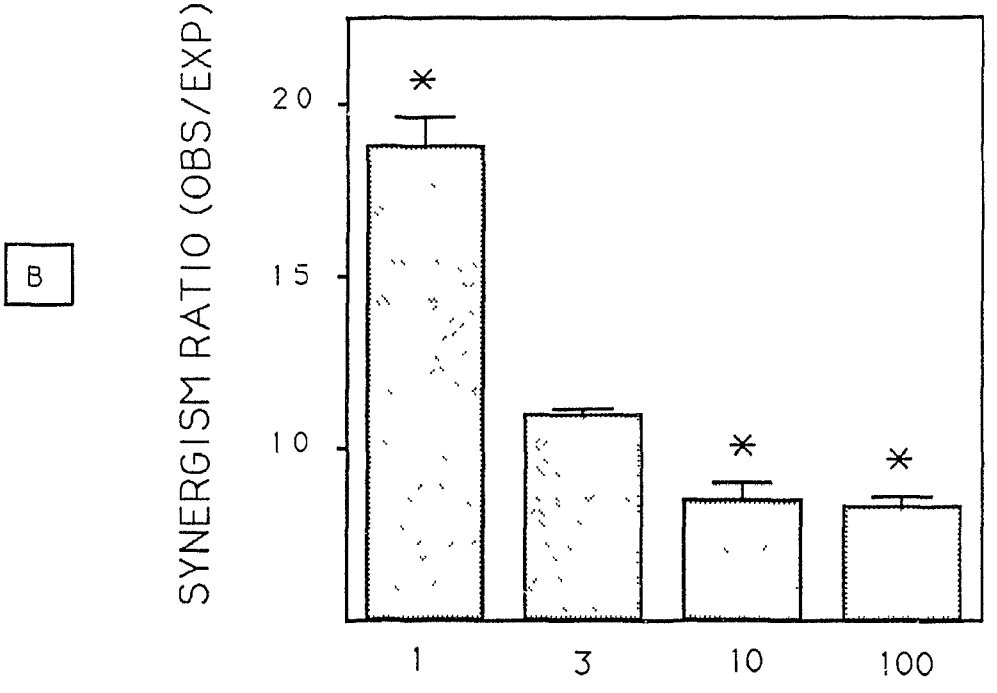

B
SYNERGISM RATIO (OBS/EXP)
20
15
10
*
*
*
1
3
10
100
[LH] (ng/ml)

Figure 15.

Calculation of synergism ratios (Fig. 15B) supported this impression, revealing a biphasic interaction such that the steroidogenic response to 1 ng/ml LH and ANF was significantly greater than an additive response while the combined response to ANF and higher LH concentrations was significantly attenuated.

2. Interaction between the site/type-selective cAMP analogues and cGMP

Having established the capabilities of the site/type-selective cAMP analogues to synergistically interact with processes mediated via cAMP (Figs. 10 and 12), the potential for cooperative interaction with the cyclic guanosine second messenger system was evaluated. As can be seen in Fig. 16A, coexposure of the cells to each of the cAMP analogues and stimulatory levels of the cGMP analogue, 8-bromo-cGMP (500 μM; control - 69.2 ± 13.3, 8-Br-cGMP-225.4 ± 69.4 ng androgen/million cells/3h) resulted in a significant synergistic elevation in androgen production seemingly via both PK-A isoenzymes. As the 8-bromo- moiety of the cGMP analogue could conceivably increase its affinity for the PK-A regulatory subunits (Lincoln and Corbin, 1983), the experiment was repeated using 2 mM cGMP which was ineffective in stimulating androgen production (control-54.1 ± 12.1 vs cGMP - 55.9 ± 10.7 ng androgen/million

Figure 16. Synergism between site/type-selective cAMP analogues and cGMP. Synergistic increase in androgen production upon coexposure of mouse Leydig cells to AHA, B or TM and A) 500 μM 8-bromo-cGMP or B) 2.0 mM cGMP in the presence of 0.1 mM MIX. * denotes a statistically significant difference from an additive response (SR = 1.0), $p \leq 0.05$. Data represents the mean ± SEM of 4-5 separate experiments.

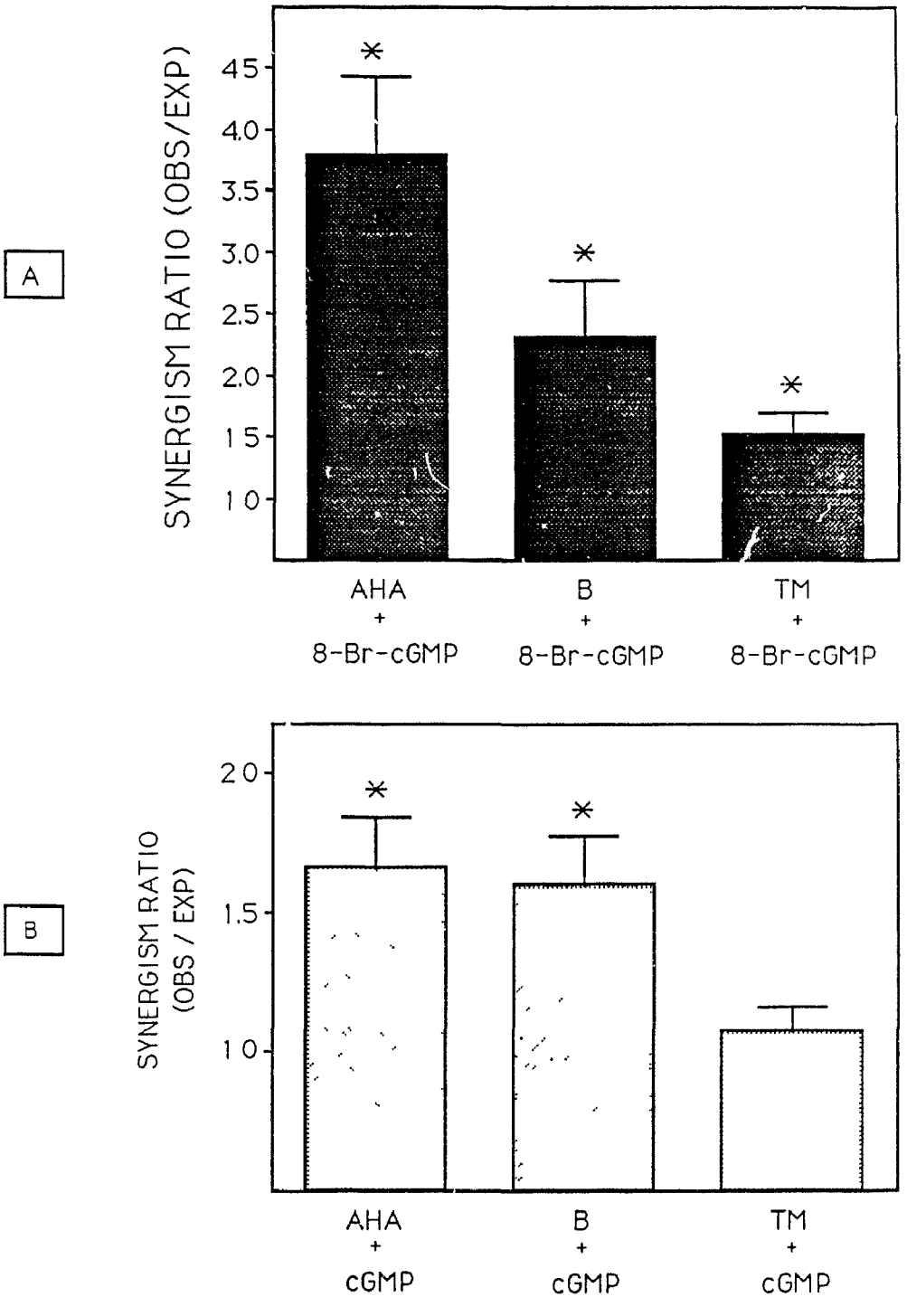

A
SYNERGISM RATIO (OBS/EXP)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
*
*
*
AHA + 8-Br-cGMP
B + 8-Br-cGMP
TM + 8-Br-cGMP
B
SYNERGISM RATIO (OBS / EXP)
2.0
1.5
1.0
*
*
AHA + cGMP
B + cGMP
TM + cGMP

Figure 16.

cells/3h). Much higher concentrations of the parent cyclic nucleotide was required due to its relative insolubility in the plasma membrane. The possibility that cGMP could elicit a synergistic interaction with the cAMP analogues through the reduction of PDE-mediated cAMP degradation was also addressed in this experiment through the inclusion of the nonspecific PDE inhibitor, MIX (0.1 mM). This concentration of MIX was appropriate for the inhibition of PDE I, II and III as its IC_{50} for these enzymes ranges from 3.4 - 12 μM (Bergstrand et al., 1977). Figure 16B illustrates that while the synergistic interaction between cGMP and the cAMP analogues remained intact, it cccurred predominantly via type 1 PK-A and was reduced considerably from that seen with 8-bromo-cGMP (Fig. 16A)

3. Interaction between the site/type-selective cAMP analogues and ANF

If exogenous cGMP can synergize with the cAMP, then a factor such as ANF which stimulates the synthesis of cGMP should also interact in a cooperative manner with the cAMP analogues. As can be seen in Fig. 17, levels of ANF which only minimally increased androgen production (1.0 - 2.5 nM; Fig. 17A), significantly enhanced the steroidogenic activation elicited by the cAMP analogues (Fig. 17B), predominantly through type 1 PK-A but also through the type

Figure 17. The steroidogenic response of Leydig cells to atrial natriuretic factor (ANF), alone or in combination with site/type-selective cAMP analogues. A) The increase in androgen production upon exposure of mouse Leydig cells to ANF (1.0-5.0 nM). B) Synergistic increase in androgen production upon coexposure of mouse Leydig cells to AHA, B or TM and ANF. * denotes a statistically significant difference from an additive response (SR = 1.0), $p \leq 0.05$. Data represents the mean ± SEM of 4-6 separate experiments.

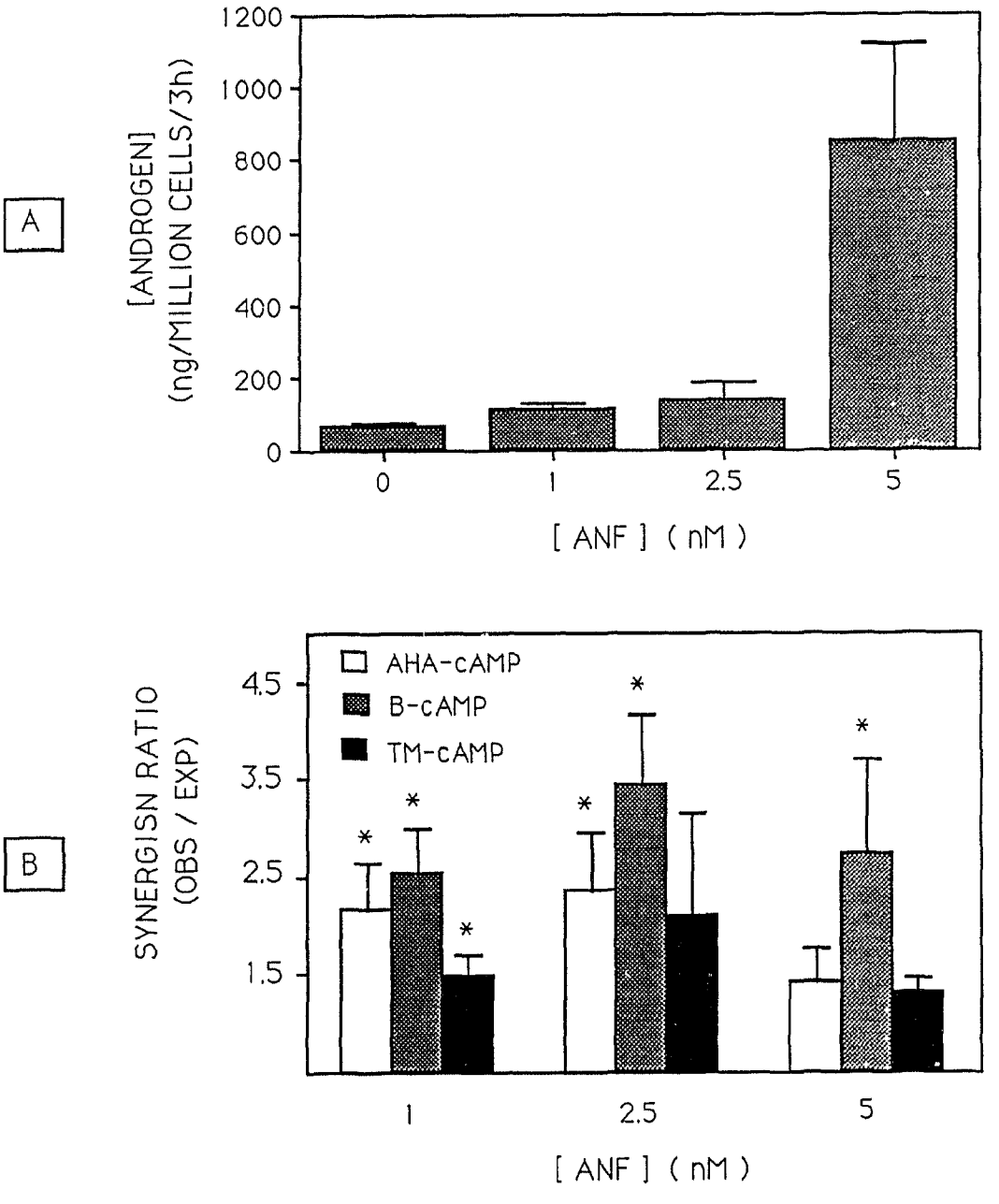

A
[ANDROGEN]
(ng/MILLION CELLS/3h)
1200
1000
800
600
400
200
0
0
1
2.5
5
[ANF] (nM)
B
SYNERGISN RATIO
(OBS / EXP)
AHA-cAMP
B-cAMP
TM-cAMP
4.5
3.5
2.5
1.5
*
1
2.5
5
[ANF] (nM)

Figure 17.

2 isoform. The S2/T1+2 analogue, B, more readily synergized with the higher concentration of ANF (5 nM), presumably due to the ability of that analogue to selectively bind with both the type 1 and type 2 PK-A isoenzymes.

Again, the interaction between the two second messenger systems does not occur at the PDE level as the inclusion of MIX augments rather than attenuates the synergistic increase in androgen production at the lower treatment levels of ANF (Fig. 18)

C. Discussion

The degree of compartmentalization or "crosstalk" between second messenger systems has not been adequately addressed due, at least in part, to the complexity of the messenger systems and the difficulties inherent in isolating them in situ. A number of studies performed shortly after the discovery of the cyclic nucleotide second messengers suggested that an adversarial relationship existed between cAMP and cGMP. These findings prompted Goldberg and coworkers (1975) to form an hypothesis based on "biologic regulation through opposing forces". This "Yin-Yang" hypothesis suggested that bidirectional regulated activities (functions which can be actively stimulated or inhibited) occurred in two ways within a cell: those stimulated by cAMP

Figure 18. The effect of PDE inhibition on the synergistic interaction between site/type-selective cAMP analogues and ANF. Synergistic increase in androgen production upon coexposure of mouse Leydig cells to ANF (1.0-5.0 nM) and A) AHA or B) B in the presence or absence of 0.1mM MIX. * denotes significant statistical difference from additive response (SR = 1.0), $p \leq 0.05$ while ** denotes a statistically significant effect of PDE inhibition. Data represents the mean ± SEM of 4 separate experiments.

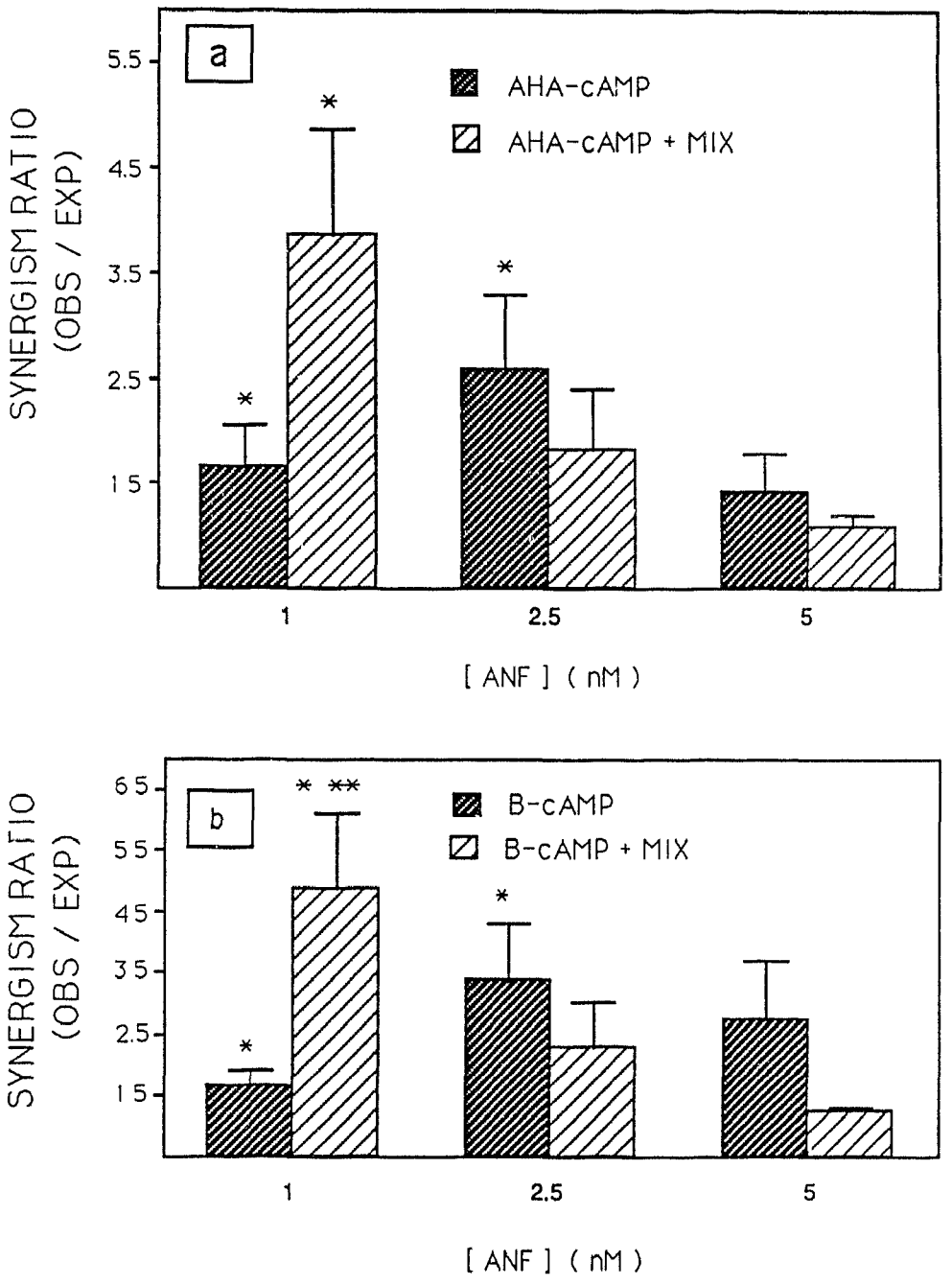

a
AHA-cAMP
AHA-cAMP + MIX
SYNERGISM RATIO
(OBS / EXP)
5.5
4.5
3.5
2.5
1.5
1
2.5
5
[ANF] (nM)
b
B-cAMP
B-cAMP + MIX
SYNERGISM RATIO
(OBS / EXP)
6.5
5.5
4.5
3.5
2.5
1.5
1
2.5
5
[ANF] (nM)

Figure 18.

and inhibited by cGMP and those which are promoted by cGMP and inhibited by cAMP. Acceptance of the Yin-Yang hypothesishas been fairly widespread and some of the early work with ANF and the murine Leydig cell supported the idea of an adversarial relationship between the two cyclic nucleotide messenger cascades. Pandey and coworkers (1985) showed that relatively high concentrations of ANF reduced gonadotropin-stimulated cAMP accumulation by Leydig tumour cells (Pandey et al., 1985) while low concentrations of ANF inhibited the steroidogenic response of both normal and tumour cells to high stimulatory levels of LH (Pandey et al., 1985, 1986b). Although Mukhopadhyay et al. (1986a) could not duplicate the ANF-mediated attenuation of gonadotropin responsiveness in the mouse Leydig cell, data in this study (Fig. 15) support an inhibitory effect of low ANF concentrations on androgen production maximally stimulated by LH. The physiological relevance of the inhibitory effect is suspect, however. Although the steroidogenic response to 10 and 100 ng/ml LH and ANF was reduced relative to the potential additive response, there was little if any effect on absolute androgen production (Fig. 15A).

All of these findings are suggestive of an adversarial relationship between cAMP and cGMP and provide support for Yin-Yang hypothesis. However, Goldberg et al. (1975) did not refer to data from cell types in which both

cAMP and cGMP positively mediated the same cellular activity. Indeed, the universality of the Yin-Yang hypothesis is questionable in light of studies which noted a positive, cooperative interaction between cAMP and cGMP in olfactory cilia and pinealocytes. Nakamura and Gold (1987) found that both cAMP and cGMP would activate current flow in patches excised for olfactory cilia while Furman and Tanaka (1989) used excised patches from rod outer segments to demonstrate that micromolar concentrations of cAMP produced a large increase in photoreceptor current in the presence of physiological levels of cGMP.

The mouse Leydig cell is another cell type in which cAMP and cGMP mediate the same process: the hormonal stimulation of androgen synthesis. While the role of LH/cAMP as the predominant stimulatory mediator of steroidogenic function in the Leydig cell is well accepted (Catt et al., 1972; Mendelson et al., 1975; Cooke et al., 1976), the potent stimulation of androgen production by ANF mediated by cGMP has just recently been established in the mouse testis (Pandey et al., 1985,1986; Mukhopadhyay et al., 1985). With both the LH/cAMP and ANF/cGMP pathways present in one cell type and driving the same cellular mechanisms, it seemed likely that the systems could act cooperatively rather than adversarially.

None of the early studies which examined the effect of coexposure of Leydig cells to ANF and LH/hCG (Pandey et

al., 1985, 1986b; Mukhopadhyay et al., 1986a,b) utilized minimally stimulatory levels of LH and ANF. In doing so, it became apparent that a cooperative synergistic interplay between the two cyclic nucleotides was possible (Figs. 15, 16 and 17).

The mechanism(s) through which LH/cAMP and ANF/cGMP cascades could cooperatively enhance androgen production is not so readily apparent, however. The possibility was addressed that either the cAMP analogues or cGMP generated via ANF stimulation induced cAMP sparing through inhibition of PDE-mediated cAMP hydrolysis (Fig. 18). If the effect was mediated in this manner, inclusion of the nonspecific PDE inhibitor, MIX (Weishaar, 1987), would be expected to reduce or abolish any synergistic increase in androgen production upon coexposure of the Leydig cells to ANF and the site/type-selective analogues. As can be seen in Fig. 18, this was not the case in that synergistic interaction between ANF and the two analogues (AHA and B) was augmented by PDE inhibition. Therefore, it would appear unlikely that the cooperative interaction between the cyclic nucleotides occurred at the level of the PDE enzyme.

The fact that PDE inhibition did not reduce the degree of cooperative interaction between cAMP and cGMP raises an interesting point of discussion regarding the heightened synergism attained with 8-bromo-cGMP (Fig. 16A) over that noted with the parent cyclic nucleotide (Fig.

16B). If the inclusion of MIX in the incubation media can be ruled out as the cause for the diminished synergism noted with cGMP, then the increase in synergistic interaction with the cAMP analogues must relate to the 8-bromo- moeity on the cGMP analogue. 8-bromo-cGMP was considerably more effective in stimulating androgen production than was cGMP, which could simply be attributed to the enhanced membrane solubility of the analogue and its increased resistance to hydrolysis. This would result in a higher intracellular concentration relative to cGMP and an increased potential for interaction with the cAMP-dependent protein kinases. However, Lincoln and Corbin (1983) established that certain cAMP/cGMP derivatives used in intact cell studies have unphysiological affinities for their own and other cyclic nucleotide-dependent kinases. For example, 8-bromo-cAMP was found to be 10-50 times more effective than cAMP in activating PK-G (Lincoln and Corbin, 1983). These findings suggest that the enhanced synergism attained with 8-Br-cGMP could arise in part from its pharmacological activities within the cell.

An alternate site for the interaction between the cAMP and cGMP second messengers could be at the site of their synthesis. However, it has been demonstrated repeatedly that ANF either had no effect or inhibited cAMP synthesis in the Leydig cell (Pandey et al., 1985, 1986b; Mukhopadhyay et al., 1986a,b; Fig. 14) and other tissues

(Anand-Srivastava et al., 1984). This would strongly suggest that the interaction between ANF and the cAMP analogues (Fig. 17) was not a result of an ANF-stimulated increase in cAMP synthesis.

The synergistic increase in androgen production upon coexposure to ANF/cGMP and LH/cAMP (Fig. 15, 16 and 17) could also be mediated through activation of a common phosphotransferase amplifier. However, the relative specificity of cAMP and cGMP for their respective protein kinases approaches two orders of magnitude (Lincoln and Corbin, 1983) and the selective cAMP analogues do not crossreact with cGMP-dependent protein kinase (Corbin et al., 1988). At first, this would seem to refute cooperative intramolecular interaction through either one of the cyclic nucleotide-dependent kinases. In fact, this low affinity binding could provide a clue to the mechanism through which interaction was achieved: autophosphorylation of the protein kinase. Both cAMP and cIMP have been shown to bind with low affinity to PK-G and stimulate PK-G autophosphorylation (deJong and Rosen, 1977), an intramolecular process which was shown to enhance kinase activity by inhibiting the ability of the regulatory domain to recombine with the catalytic subunit (Rosen et al., 1977). Lincoln and Corbin (1983) speculated that this low affinity binding converted PK-G to an intermediate form which was more readily autophosphorylated. The cAMP

analogues could act in a similar manner to increase the rate or degree of PK-G autophosphorylation. Alternatively, cGMP could be "priming" the PK-A isoenzymes for autophosphorylation and in doing so, enhance the effects of the cAMP analogues in stimulating steroidogenesis. This would seem unlikely, however, as ANF/cGMP synergism with the type 2 PK-A (Fig. 13 and 14), the isoform most prone to autophosphorylation (Rosen and Erlichman, 1975), was not predominant. However, as type 2 PK-A has been shown to exist primarily in a phosphorylated state (Rangel-Aldao et al., 1979), it may be less sensitive to further autophosphorylation.

There is evidence that the two species of protein kinases could interact in an intermolecular fashion to cooperatively enhance steroidogenic activation. For example, a large degree of substrate overlap between the two kinases has been demonstrated in other cell types (Lincoln and Corbin, 1983). However, phosphorylation of common substrates could be expected to additively rather than synergistically enhance the ability of either protein kinase to support steroidogenic activities. The phosphorylative state of the cyclic nucleotide-dependent protein kinases may also be influenced in an intermolecular fashion. Geahlan and Krebs (1980) demonstrated that the purified regulatory subunit of type 1 PK-A was phosphorylated by PK-G at a site which was in the trypsin-sensitive area involved in R-C

interaction but independent of that involved in autophosphorylation (Hashimoto et al., 1981). It was subsequently shown *in vitro* that this phosphorylation reduced the inhibitory effect of the regulatory subunit on the activity of the C subunit ie. analogous effect to autophosphorylation (Geahlen et al., 1981). This could represent the mechanism of action through which cGMP could synergistically enhance the steroidogenic effect of the cAMP analogues and account for the tendency for cGMP/ANF to interact with the type 1 PK-A isoenzyme (Figs. 15, 16 and 17). However, the physiological relevance of type 1 PK-A phosphorylation by PK-G has not been established. The rate of phosphorylation is very slow (Gaehlan and Krebs, 1980) and although it alters the interaction of the regulatory and catalytic subunits *in vitro*, it does not appear to do so *in vivo* (Geahlen et al., 1981). Further studies are necessary to clarify the role of this process in the mouse Leydig cell.

A provocative possibility for a site of interaction between cyclic nucleotides would be through a common protein kinase which specifically binds both cAMP and cGMP. Such an enzyme has been identified and partially characterized in the grasshopper by Vardanis (1980) as a homodimer which binds both cAMP and cGMP with similar and relatively high affinities (43 and 25 nM, respectively). Although isolated from an insect, it is not out of the realm of possibility

that a similar species of protein kinase could exist in mammalian cells. The possibility that a cAMP/cGMP-dependent phosphotransferase may be present in the murine Leydig cell certainly warrants further investigation.

In conclusion, it is apparent that LH and ANF and their respective cyclic messenger systems, cAMP and cGMP have the capability to interact in a positive manner to stimulate androgen production. It is not known whether this interaction occurs via one of the cyclic nucleotide-dependent protein kinases, through an as of yet unidentified cAMP/cGMP-dependent kinase or by some other mechanism.

V. STUDY 3

A novel inhibitory function for calmodulin in the mouse Leydig cell: the possible involvement of an inhibitory lipoxygenase system

A. Introduction

Having reaffirmed the importance of the cyclic nucleotide second messenger networks in mediating external control of steroidogenic activity in the Leydig cell, it should be noted that other putative second messenger systems have been identified in the murine Leydig cell including intracellular calcium (Janszen et al., 1976; Sullivan and Cooke, 1986) and the eicosanoids (Dix et al., 1984; Lin, 1985; Abayasekara et al., 1990).

The ubiquitous calcium-binding protein, calmodulin (CaM), was originally shown to play a role in rat Leydig cell steroidogenic function by Hall and coworkers (1981). They demonstrated that introduction of Ca^{2+}/CaM into incubated Leydig cells with liposomes stimulated both basal and LH-stimulated androgen production while exposure to the CaM inhibitor, trifluoperazine (TFP) inhibited both basal steroidogenesis and the ability to respond to LH. Sullivan and Cooke (1985) also found that relatively high levels of the CaM antagonist, calmodazolium, inhibited rat Leydig cell steroidogenic function, again in static incubations. It is

interesting to note that in this same study, low concentrations of calmodazolium actually enhanced the response of rat Leydig cells to gonadotropin-releasing hormone (GnRH) (Sullivan and Cooke, 1985). This supported some preliminary incubation studies in this laboratory using murine Leydig cells in which low concentrations of both TFP (P.R. Murphy, unpublished observations) and another CaM antagonist, N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W7) (Tanaka et al., 1982; MacNeil et al., 1988) elicited an interesting though inconsistent stimulatory effect on androgen production. We realized that the static nature of our incubation technique (i.e. measurement of total accumulation of testosterone over 3h) could be confounding our attempts to distinguish a biphasic response mediated by CaM antagonism. In an attempt to better differentiate and characterize this W7 response, we developed a multi-chambered cell perifusion system which allowed for the sequential monitoring of androgen production over a period of hours following a transient exposure to W7.

In fact, exposure to W7 did elicit a biphasic effect comprised of an initial inhibition followed by a dose-dependent stimulation of androgen production. There was indirect evidence that the stimulation of steroidogenic function was mediated by an arachidonic acid following the inhibition of a calmodulin-sensitive lipoxygenase enzyme.

B. Results

1. The effect of W7 on androgen production in Leydig cell incubations

Incubation of Leydig cells (20,000 cells/500 μl TC-199 for 3h) with W7 (0-200 μM) elicited an inconsistent stimulatory steroidogenic response at lower W7 concentrations and a more consistent inhibitory effect with the 200 μM concentration (Fig. 19). The difficulty in isolating an initial stimulatory response in static incubation led to the development of a multi-chambered cell perifusion system (Fig. 6).

2. Treatment dilution during cell perifusion

Due to the nature of the perifusion system, there was an inherent potential for dilution during short term treatment infusions. To confidently establish that any potential steroidogenic response to W7 was mediated through specific antagonism of CaM, that response should occur at concentrations of the drug which have been shown to specifically inhibit CaM-sensitive systems ($IC_{50} \approx 30$ μM, MacNeil et al., 1988). Therefore, it was necessary to accurately measure the intrachamber W7 concentration by

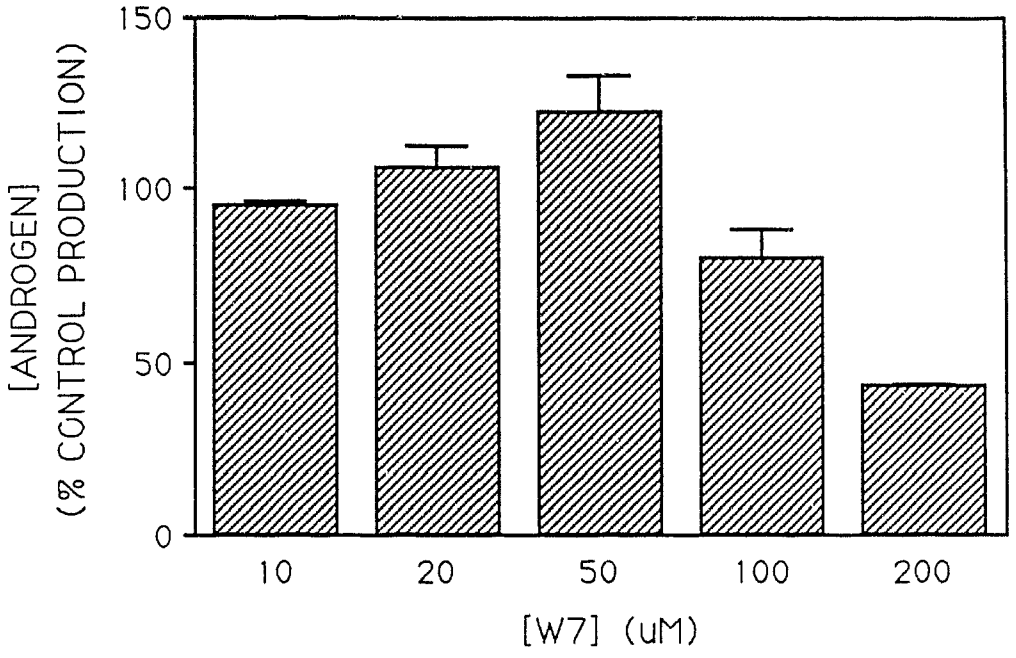


Figure 19. The effect of W7, a calmodulin antagonist, on androgen production by incubated mouse Leydig cells. Cells were exposed to 0-200 μM W7 during 3h static incubations as described in **Materials and Methods**. Data is expressed as a percentage of control production. All data represents means ± SEM of 4 separate experiments.

collecting the perifusate immediately as it exits the chamber. MacNeil and coworkers (1988) noted that iodonapthalenesulfonamides had a marked fluorescence (maximum excitation = 293 nm, maximum emission = 345 nm). Preliminary experiments with W7 demonstrated that the chlorine-substituted derivative was also very fluorescent with similar excitation/emission properties (310 nm and 375 nm, respectively). The fluorescent nature of W7 provided a simple and direct method for measuring the levels of the CaM antagonist in the perifusate collected immediately upon its passage through the perifusion chamber. Duplicate perifusion systems were infused with 100 μM W7 for 20 min; the perifusate was collected at 10 min intervals beginning at time 0 and the fluorescence of the media was measured with a Perkin-Elmer spectrofluorometer. Extrapolation of fluorescence readings against a standard curve (0-200 μM W7; Fig. 20A) revealed that there was a minimum 40 % dilution of the 100 μM W7 (Fig. 20B). Although W7 was infused for only 20 minutes, the cells were exposed to the CaM antagonist for approximately 80 min beginning 30 min following the onset of infusion. The maximal intrachamber concentration of W7 (60 μM) occurred $\approx$ 60 min after the infusion was initiated (Fig. 20B).

Figure 20. The effects of post-infusion dilution on the intrachamber W7 concentration during perifusion experiments. Fig. 20A. The fluorescent emissions of W7 (0-200 μM) were measured following the establishment of maximally effective excitation (310 nm, slit width 10 μm) and emission wavelengths (375 nm, slit width 5 μm). Fig. 20B. Duplicate perifusion systems were infused with 100 μM W7 for 20 min beginning at 0 min. The concentration of W7 in the perifusate fractions (collected at 10 min intervals) was assessed by comparing the strength of the samples fluorescent signal to that emitted from the standards (Fig. 20A). Although the system did not contain Leydig cells, experimental conditions were otherwise identical to other perifusion studies.

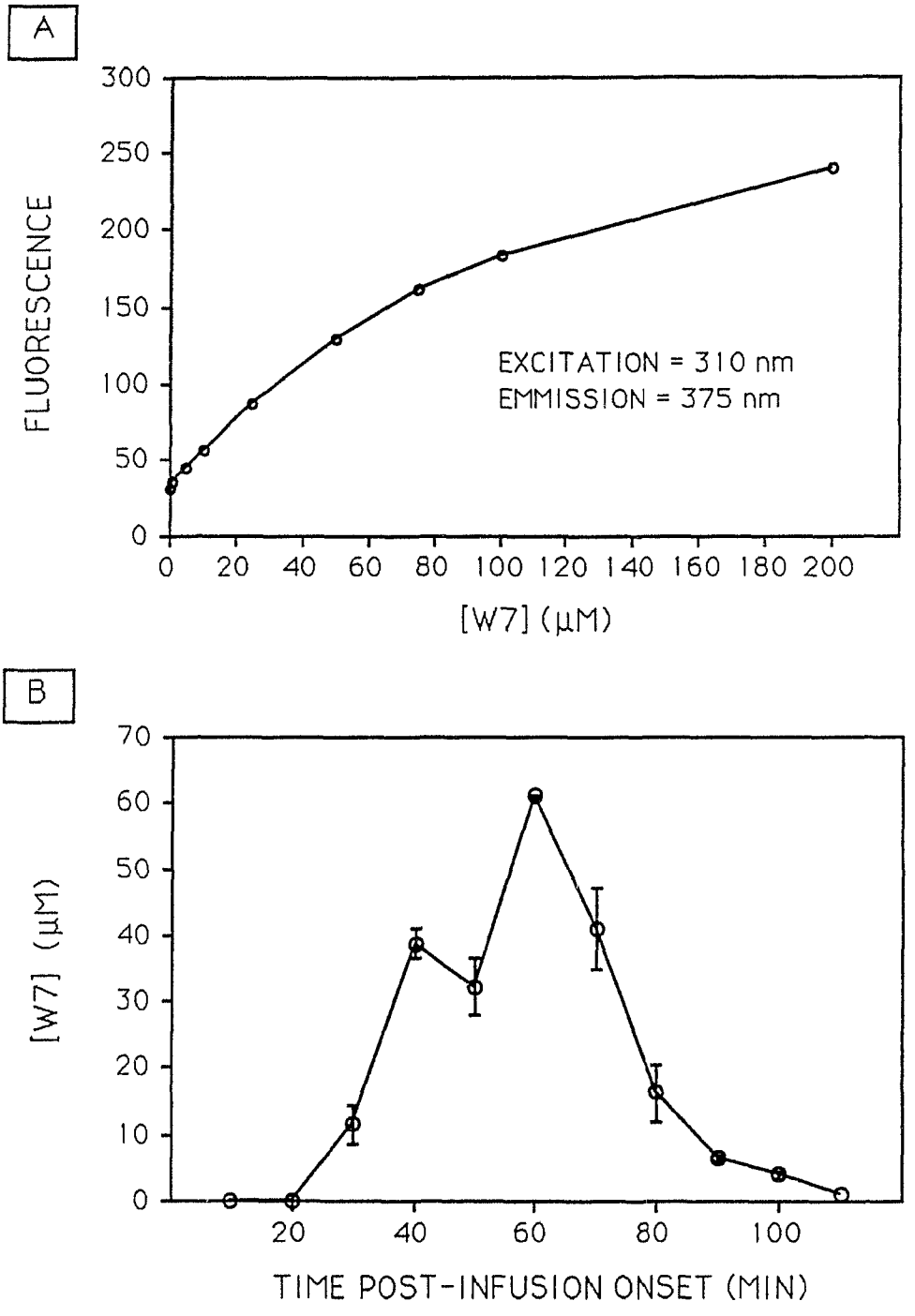

A
FLUORESCENCE
300
250
200
150
100
50
0
0 20 40 60 80 100 120 140 160 180 200
[W7] (μM)
EXCITATION = 310 nm
EMMISSION = 375 nm
B
[W7] (μM)
70
60
50
40
30
20
10
0
20 40 60 80 100
TIME POST-INFUSION ONSET (MIN)

Figure 20.

3. The effect of W7 on androgen production by perifused Leydig cells

As shown in Fig. 21A, cell perifusion revealed that a 20 min infusion of 100 μM W7 elicited a biphasic effect on androgen production comprised of an initial inhibitory response (at 90 min, control - 1.01 ± 0.08 vs W7- 0.16 ± 0.03 ng/million Leydig cells/10 min; $p \leq 0.0001$) followed by a stimulatory effect which peaked at 160 min (control - 0.67 ± 0.04 vs W7- 4.68 ± 0.75 ng/million Leydig cells/10 min; $p \leq 0.0001$). The degree of W7 stimulation at its maximum (at ≈160 min; Fig. 21A) was approximately 12% of that which could be achieved upon exposure to highly stimulatory levels of LH (100 ng/ml for 5 min; Fig. 25). Infusion of 20 μM W7 did not stimulate androgen production though the inhibitory effect at 90 min was equivalent to that seen with 100 μM W7 (Fig. 21B; at 90 min, 20 μM W7- 0.17 ± 0.04 ng/million Leydig cells/10 min; $p = 0.84$). It was also apparent that the stimulatory response to 100 μM W7 commenced before the inhibitory effects of the 20 μM concentration had subsided (at 100-110 min, Fig 21B).

Dose response studies (Fig. 22) revealed that even W7 concentrations as low as 5 μM significantly inhibited androgen production as assessed by a Scheffe F-test (control- 0.94 ± 0.10, 5 μM W7- 0.47 ± 0.03 ng/million Leydig cells/10 min). Both 50μM and 100 μM W7 elicited the

Figure 21. The effect of W7 on androgen production by perifused mouse Leydig cells. A) Cells were perifused with 0, 20 or 100μM W7 for 20 min, resulting in an exposure of the cells to W7 from 80-160 min (as indicated by the shaded bar) (see Fig. 20B) following a 70 min baseline period as described in the **Materials and Methods**. Data points represent means ± SEM of 7 separate experiments. B) Details of the initial inhibition of androgen production noted upon W7 infusion in Fig. 21A. Note the identical degree of inhibition of androgen production at 90 min by cells exposed to either 20 μM and 100μM W7. * denotes a statistically significant difference ($p \leq 0.004$) relative to androgen production from the control chamber.

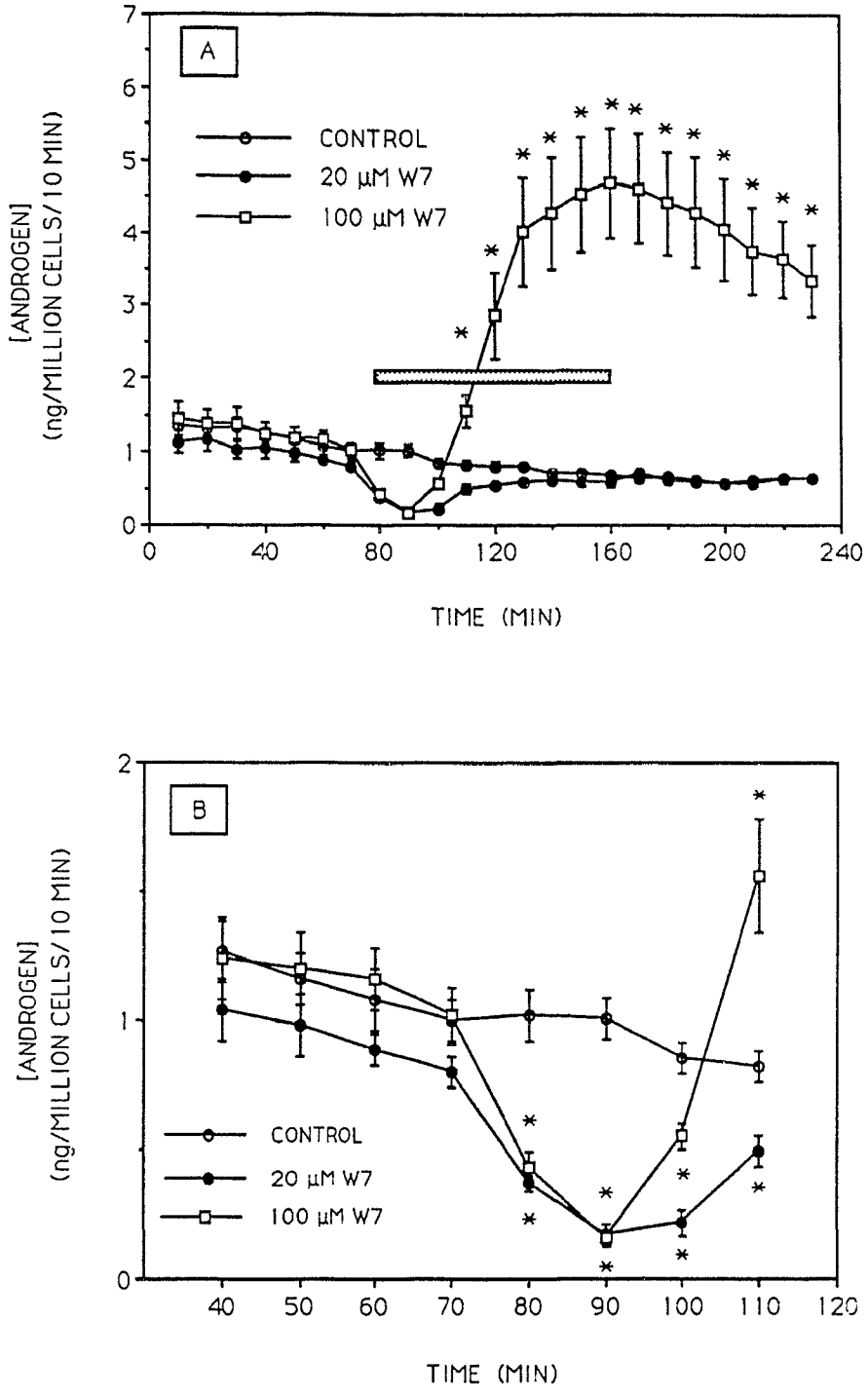

A
CONTROL
20 µM W7
100 µM W7
[ANDROGEN]
(ng/MILLION CELLS/10 MIN)
TIME (MIN)
B
CONTROL
20 µM W7
100 µM W7
[ANDROGEN]
(ng/MILLION CELLS/10 MIN)
TIME (MIN)

Figure 21.

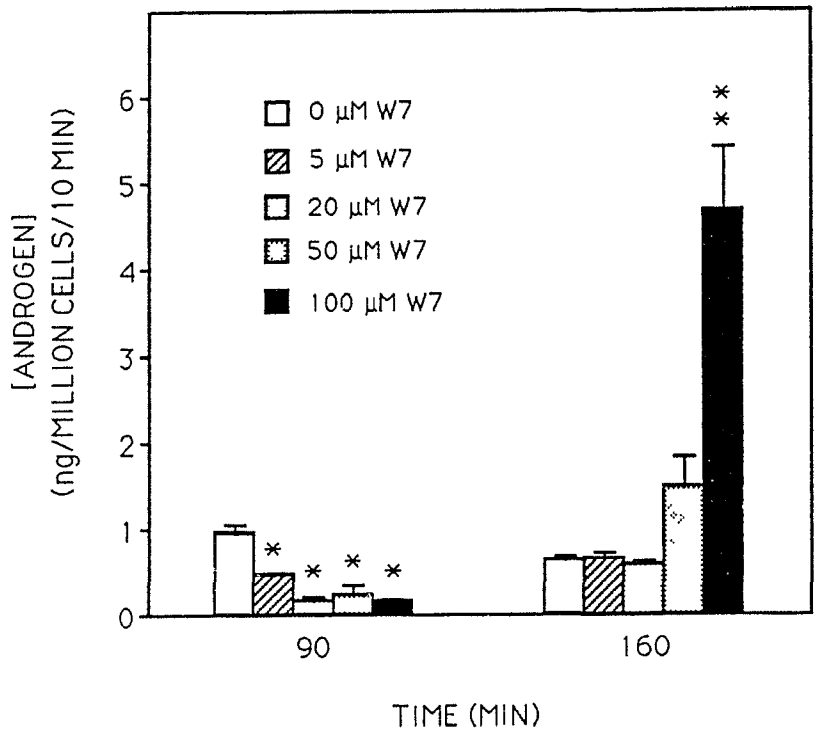


Figure 22. The effect of W7 concentration on androgen production by perifused Leydig cells. The effect of perifusion (from 80-160 min) with various concentrations of W7 (0, 5, 20, 50 and 100 μM) on androgen production at 90 and 160 min of the perifusion. Data represents means ± SEM of 5-8 separate experiments. * denotes statistical significance ($p \leq 0.0006$) relative to the control chamber. Double star denotes a statistically significant difference ($p \leq 0.0006$) relative to the effect of the previous W7 concentration.

subsequent stimulatory effect in a dose-dependent manner though the response to 50 μM W7 was not statistically significant (at 160 min, control- 0.62 ± 0.06; 50 μM W7- 1.50 ± 0.33; 100 μM- 4.69 ± 0.75 ng androgen/million cells/ml).

4. The calmodulin-specificity of the W7 effects

To assess whether the effects of W7 were specifically due to calmodulin antagonism, some of the experiments were repeated using the dechlorinated W7 analogue, W5, which has been demonstrated to be a very weak CaM antagonist (Tanaka et al., 1982). As can be seen in Fig. 23, transient exposure to 100 μM W5 also significantly inhibited androgen production relative to that in control chambers (control- 0.83 ± 0.10; W5- 0.12 ± 0.050; $p \leq 0.0008$). While the inhibitory effect of W5 was equivalent to that seen with 100 μM W7 (at 90 min, $p = 0.14$), there was no subsequent stimulation by 100 μM W5 ($p \leq 0.55$), indicating that the W7-induced stimulation was a CaM-dependent effect. The dilution study (Fig. 20) would also support a CaM-dependent stimulatory effect. Although the cells were infused with 50-100 μM W7, (Figs. 21 and 22), dilution resulted in intrachamber W7 concentrations closer to 30-60 μM. These W7 concentrations were appropriate for antagonism of CaM-dependent events (MacNeil et al., 1988).

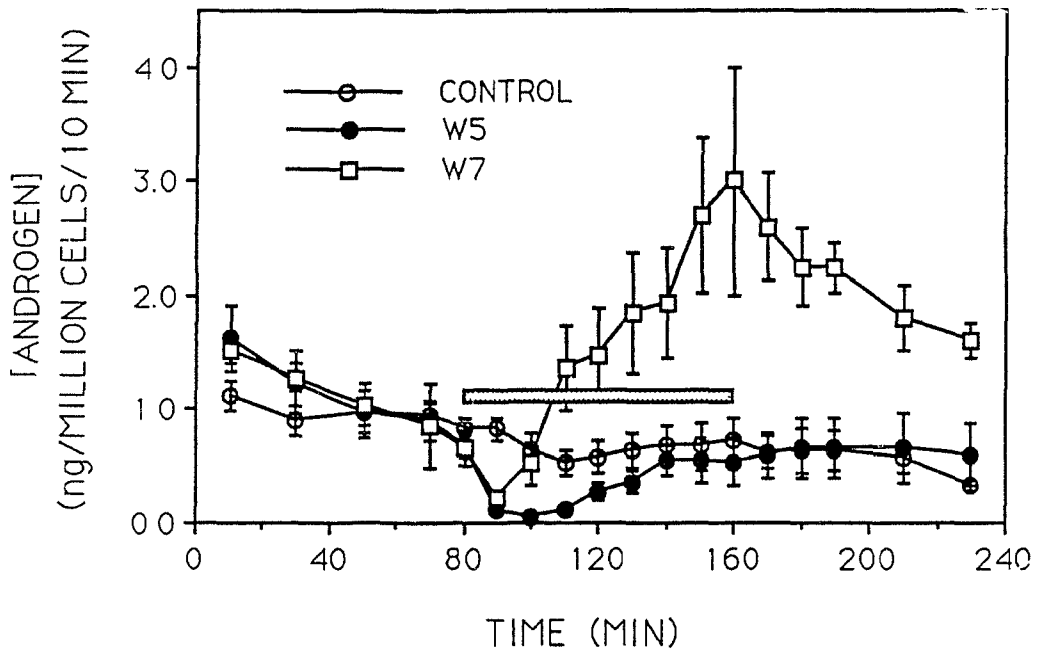


Figure 23. The effect of W7 and its inactive analogue, W5, on androgen production by perifused mouse Leydig cells. The cells were perifused with media, 100μM W7 or 100μM W5 (from 80-160 min, as indicated by the shaded bar). Data represents means ± SEM of 4 separate experiments.

As even low treatment levels of W5 initially inhibited androgen production (control- 0.85 ± 0.06 vs 5 μM W5- 0.42 ± 0.08; n =4), it would appear that the inhibitory response was a side effect of the napthalenesulphonamide drugs independent of any anti-calmodulin activity.

5. The role of cholesterol delivery on the inhibitory response to W7

Hall and coworkers (1981) demonstrated that CaM antagonism reduced the delivery of cholesterol to the steroidogenic pathways located in the inner mitochondrial membranes. We examined the role of cholesterol delivery in the initial inhibitory effect of W7. Cells in two chambers were chronically infused with perifusate augmented with 1 μM 25-hydroxycholesterol (25-OH-chol), which does not require carrier-mediated access to the mitochondrial membranes (Sullivan and Cooke, 1984). As can be seen in Table II, although 25-OH-chol significantly enhanced androgen production, there was no significant effect on the initial inhibitory response to 100 μM W7. This strongly suggests that the inhibitory effect of the napthalenesulfonamides occurs at a point beyond the delivery of cholesterol to the $P450_{scc}$ in the mitochondria.

Table II. The effect of exogenous 25-hydroxycholesterol on the inhibitory effect of 100 μM W7 on androgen production by perifused Leydig cells.

TREATMENT	[ANDROGEN] (ng/MILLION CELLS/10 MIN)[1] 90 MIN	
BASAL	0.82 ± 0.17	
BASAL + W7	0.27 ± 0.08	
25OH-chol	4.50 ± 0.81	*
25OH-chol+ W7	0.30 ± 0.09	**

[1] Mean ± SEM of 4 separate experiments.
* $p \leq 0.01$ relative to basal production.
** $p \leq 0.01$ relative to 25-OH-chol control

6. The effect of W7 on calmodulin-sensitive phosphodiesterase activity

It was possible that W7 could have been increasing androgen production by delaying the degradation of cyclic nucleotide second messengers, cAMP and/or cGMP via the inhibition of a CaM-dependent cyclic nucleotide PDE enzyme (Levin and Weiss, 1977). We addressed the possibility of messenger sparing by assessing if inclusion of a phosphodiesterase inhibitor (0.1 mM MIX) in the perifusate effected the response to W7 and by directly measuring cAMP and cGMP in the effluent.

Measurement of cyclic nucleotide levels in the effluent was not particularly informative. Although the inhibitory and stimulatory effects of W7 were not associated with any significant change in either cAMP or cGMP levels (Table III and IV) neither was the highly stimulatory effect of LH (Table IV) which has been shown to increase extracellular cAMP in incubations (Dufau et al., 1977). However, as can be seen in Fig. 24, tonic inhibition of PDE with MIX affected neither the inhibitory nor the stimulatory steroidogenic response to 100 μM W7 relative to the control responses, suggesting that neither cAMP/cGMP sparing nor synthesis is involved in the effects of W7.

Table III. The effect of W7 on cAMP levels in perifusion effluent at 90 min.

TREATMENT	90 MIN [cAMP]* (pmol/ml)
CONTROL	7.28 ± 1.90
100 μM W7	5.01 ± 1.72

* Mean ± SEM of 7 separate experiments

Table IV. The effect of W7 and LH on cAMP and cGMP levels in perifusion effluent at 160 min.

TREATMENT	160 MIN [cAMP]* (pmol/ml)	[cGMP]* (pmol/ml)
CONTROL	5.66 ± 1.39	0.40 ± 0.19
100 μM W7	4.99 ± 1.31	0.23 ± 0.10
100 ng/ml LH	6.96 ± 2.02	-

* Mean ± SEM of 6-11 separate experiments.

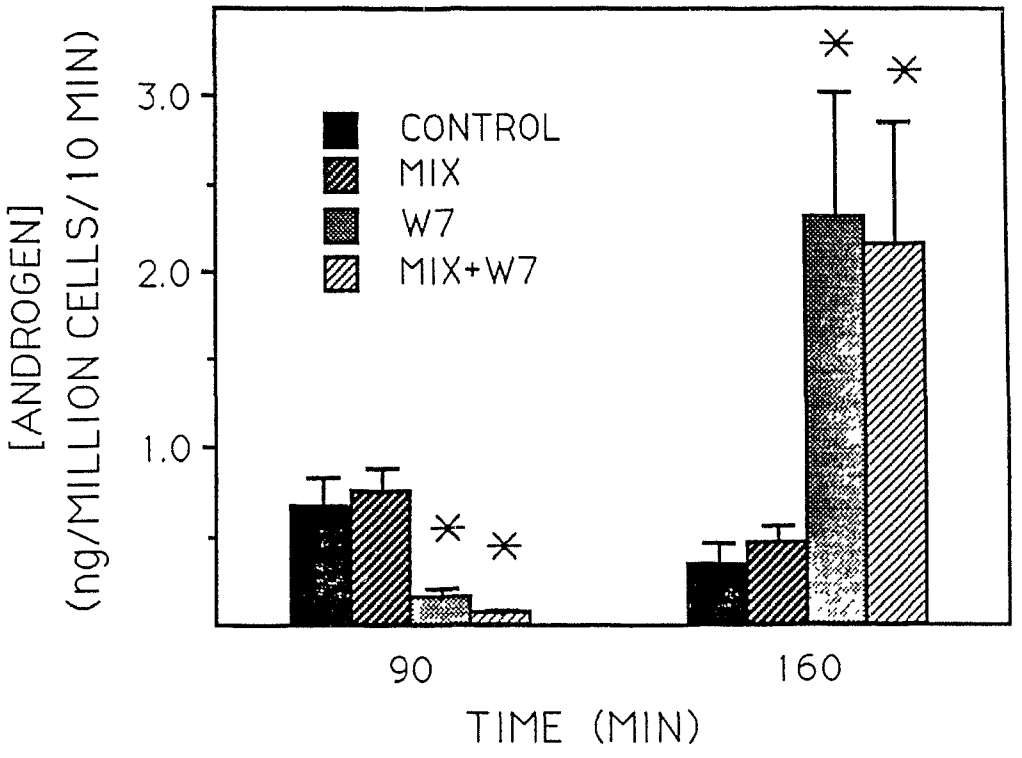


Figure 24. The effect of phosphodiesterase inhibition on the steroidogenic response to W7 by perifused Leydig cells. Cells were chronically perifused with media ± 0.1 mM MIX and transiently perifused with 0 or 100 μM W7 (from 80-160 min). Androgen production at 90 and 160 min of the perifusion is depicted. Data represents means ± SEM of 5 separate experiments. * denotes a statistically significant difference ($p \leq 0.05$) relative to production from the control chamber.

7. The effect of W7 on LH-stimulated androgen production by perifused Leydig cells

As illustrated in Fig. 25, 100μM W7 significantly inhibited ($p \leq 0.02$) the steroidogenic response of perifused Leydig cells to a 5 min pulse of 100 ng/ml LH timed to stimulate steroidogenesis coincidently with maximal W7-mediated stimulation (160 min; as described in the Materials and Methods section). The response of Leydig cells to LH (control- 0.83 ± 0.16; LH- 25.8 ± 1.08; $p < 0.0001$) was not significantly inhibited, however, by co-exposure to the inactive analogue W5 (100 μM W5/LH- 22.84 ± 0.68; $p = 0.08$). This would suggest that calmodulin has a specific supportive role in the steroidogenic response to LH in mouse Leydig cells. This is consistent with earlier published reports on the rat Leydig cell (Hall et al., 1981; Sullivan and Cooke, 1985).

8. The effect of phospholipase A_2 inhibition on the stimulatory response to W7

A number of studies in a variety of cell types have noted that the activity of phospholipase A_2 (PLA_2), the rate limiting enzyme in the production of the eicosanoid cascade, was calmodulin-sensitive (Wong and Cheung,1979; Whorton et al., 1984; Craven and DeRubertis, 1985; Nakagawa and Waku, 1988). With this in mind, we supplemented the perifusion

Figure 25. The effect of W7 or W5 on LH-stimulated androgen production by perifused Leydig cells. The cells were perifused with media, 100μM W7 or 100μM W5 (from 80-160 min) and subsequently infused with 100 ng/ml LH for 5 min (beginning at 145 min) so that maximal LH response coincided with the maximal W7 stimulation (≈160 min). Data represents androgen production (mean ± SEM) at 160 min of 3 separate experiments. * denotes a statistically significant difference ($p \leq 0.0001$) relative to production from the control chamber. ** denotes a statistically significant difference ($p \leq 0.02$) relative to the response to LH.

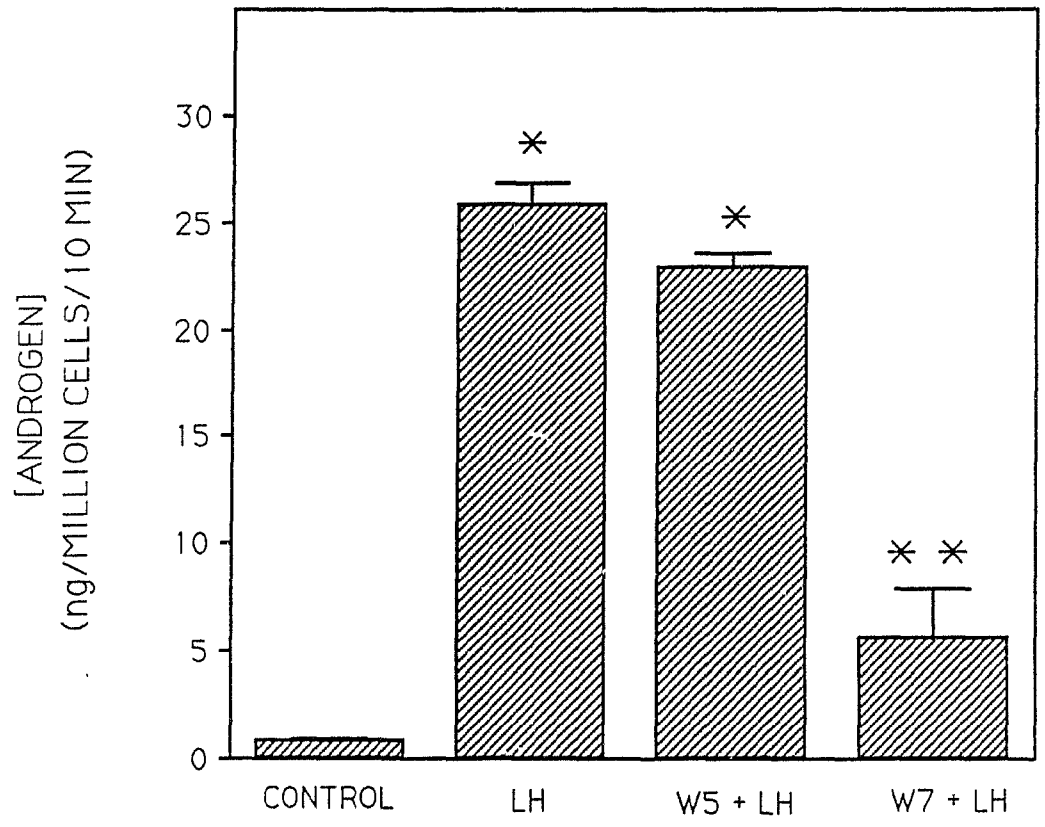

[ANDROGEN]
(ng/MILLION CELLS/10 MIN)
30
25
20
15
10
5
0
*
*
* *
CONTROL
LH
W5 + LH
W7 + LH

Figure 25.

media with mepacrine (20 μM), a specific PLA_2 inhibitor (Blackwell et al., 1977) to chronically suppress PLA_2 prior to exposure to 100μM W7. As can be seen in Table V, mepacrine virtually abolished the W7 stimulatory effect on androgen production. However, transient exposure to mepacrine in a manner analogous to the W7 perifusions (Figs 21, 22 and 23) while initially inhibiting androgen production at 90 min, did not mimic the subsequently stimulatory effect of the CaM antagonist (Table VI).

Therefore, the inhibition of a CaM-dependent PLA_2 was not mediating the stimulatory response to W7. However, arachidonic acid (AA) release appears to be required for the stimulation of steroidogenesis by W7. This would suggest that W7 was interfering with the activity of an inhibitory, calmodulin-sensitive enzyme(s) farther downstream within the eicosanoid pathway. The first criterion for identifying that enzyme would be the stimulation of androgen production upon its inhibition.

9. The effect of cyclooxygenase and lipoxygenase inhibition on androgen production

To assess the role of the different eicosanoid pathways in testicular steroidogenesis, Leydig cells were incubated with acetylsalicylic acid (ASA; 0.01 - 1 mM), a specific inhibitor of the cyclooxygenase enzyme (Pace-Asciak

Table V. The effect of chronic PLA_2 inhibition with mepacrine on the stimulatory effect of 100 μM W7 on androgen production by perifused Leydig cells.

TREATMENT	[ANDROGEN] (ng/MILLION CELLS/10 MIN)* 160 MIN	
CONTROL	1.32 ± 0.03	
CONTROL + W7	14.6 ± 1.08	**
20 μM MEPACRINE	0.52 ± 0.05	
20 μM MEPACRINE + W7	0.40 ± 0.07	

* Mean ± SEM of a representative experiment.
** $p \leq 0.05$ relative to control.

Table VI. The effect of transient PLA_2 inhibition with mepacrine on androgen production by perifused Leydig cells.

TREATMENT	[ANDROGEN] (ng/MILLION CELLS/10 MIN)1 90 MIN	160 MIN
CONTROL	0.91 ± 0.001	0.68 ± 0.09
20 μM MEPACRINE	0.37 ± 0.15 *	0.36 ± 0.02

[1] Mean ± SEM of 2 separate experiments.
* $p \leq 0.05$ relative to 90 MIN CONTROL.

and Smith, 1983) or nordihydroguaiaretic acid (NDGA; 1-100 μM), a specific lipoxygenase inhibitor (Tappel et al., 1953). There was no indication of any steroidogenic response to ASA (Fig. 26). Only exposure to 20-50 μM NDGA elicited any significant increase in androgen production in static incubations ($p \leq 0.05$; Fig. 27). This agreed with findings in the rat Leydig cell in which NDGA stimulated basal and cholesterol/pregnenolone supported steroidogenesis (Dix et al., 1984; Sullivan and Cooke, 1985) while ASA had no effect androgen production (Dix et al., 1984).

10. The steroidogenic response of perifused Leydig cells to NDGA

Figure 28 demonstrates that infusion of perifused Leydig cells with 50 μM NDGA in a manner equivalent to the W7 treatments (a 80 min pulse beginning at the 80 min point of the experiment) resulted in a steroidogenic response which was somewhat analogous to that noted with 100 μM W7 (Fig. 21A). Although the inhibitory effect was not significant, androgen production was significantly stimulated within 50 min following the initiation of NDGA exposure ($p \leq 0.05$). The degree of stimulation elicited by the NDGA treatment approached that seen with 100 μM W7 exposure ie. $\approx$ 3-4 fold increase over control production (Figs. 21A, 22 and 23).

Preliminary experiments in which the cells were either co-

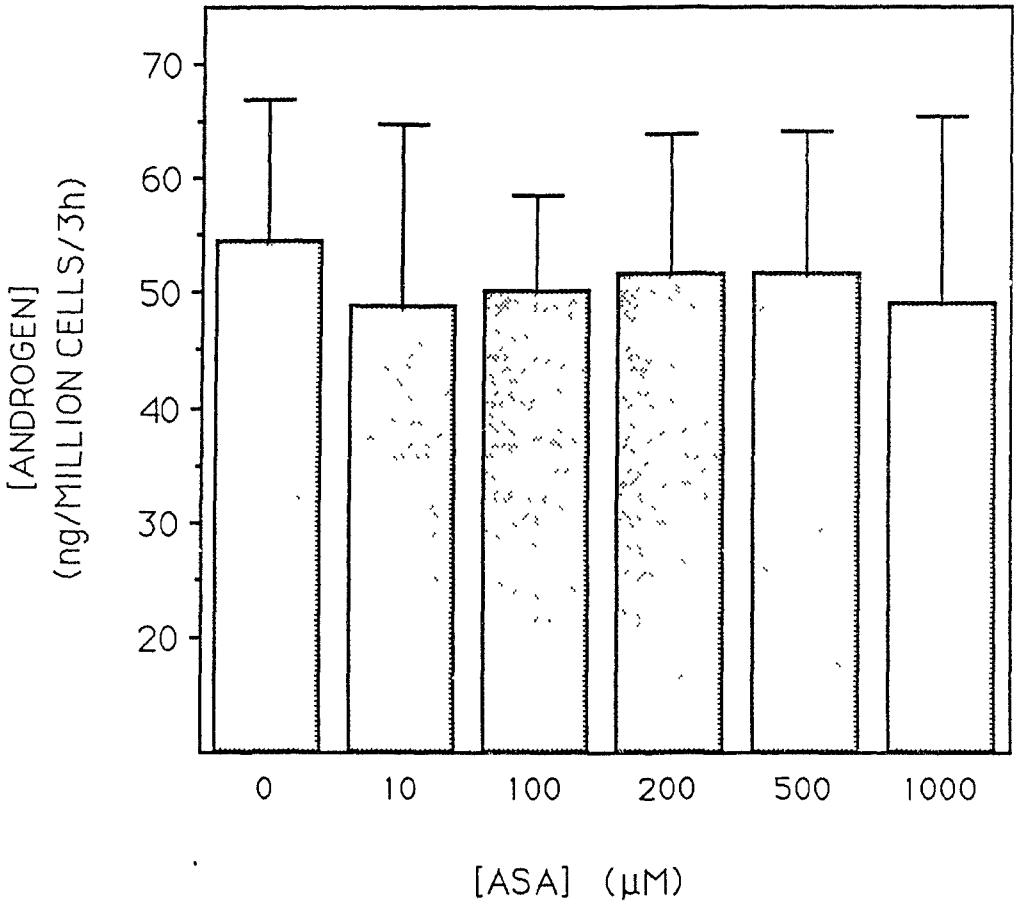


Figure 26. The effect of cyclooxygenase inhibition on androgen production in Leydig cell incubations. Cells were exposed to 0-1 mM ASA during 3h static incubations as described in **Materials and Methods**. All data represents means ± SEM of 3 separate experiments. There was no statistically significant effect on androgen production.

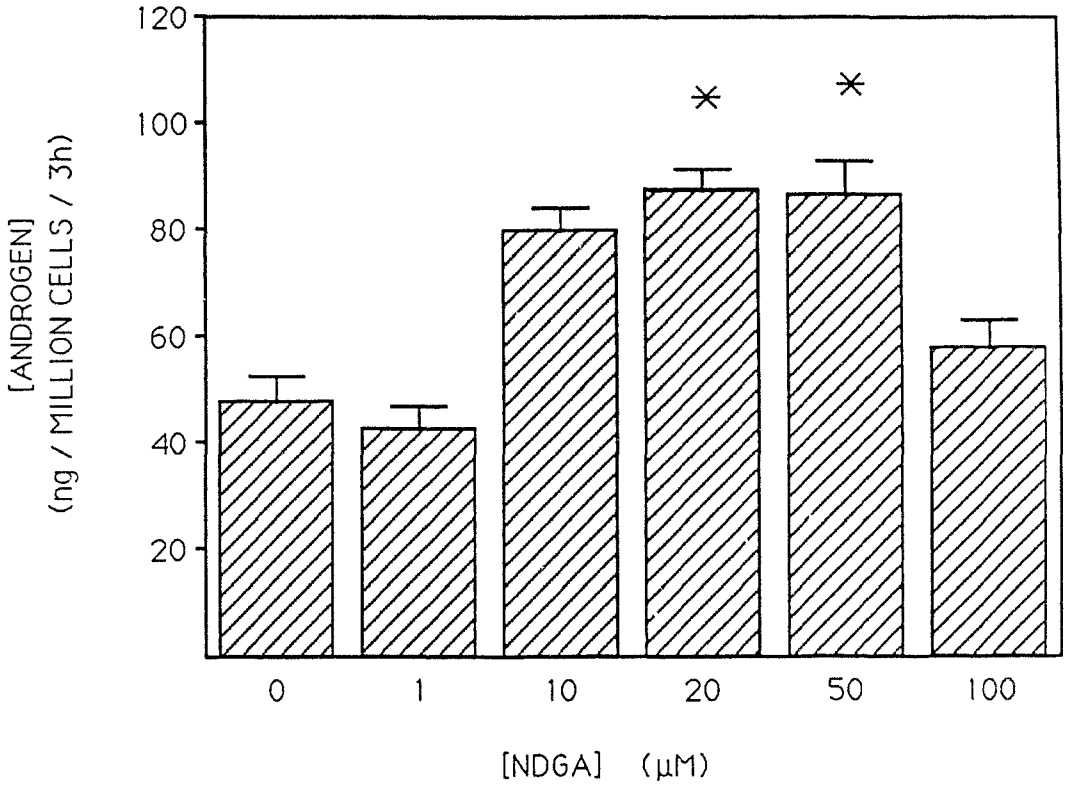


Figure 27. The effect of lipoxygenase inhibition on androgen production in Leydig cell incubations. Cells were exposed to 0-100 μM NDGA during 3h static incubations as described in **Materials and Methods**. All data represents means ± SEM of 5 separate experiments. * denotes a statistically significant difference ($p \leq 0.05$) relative to the control production.

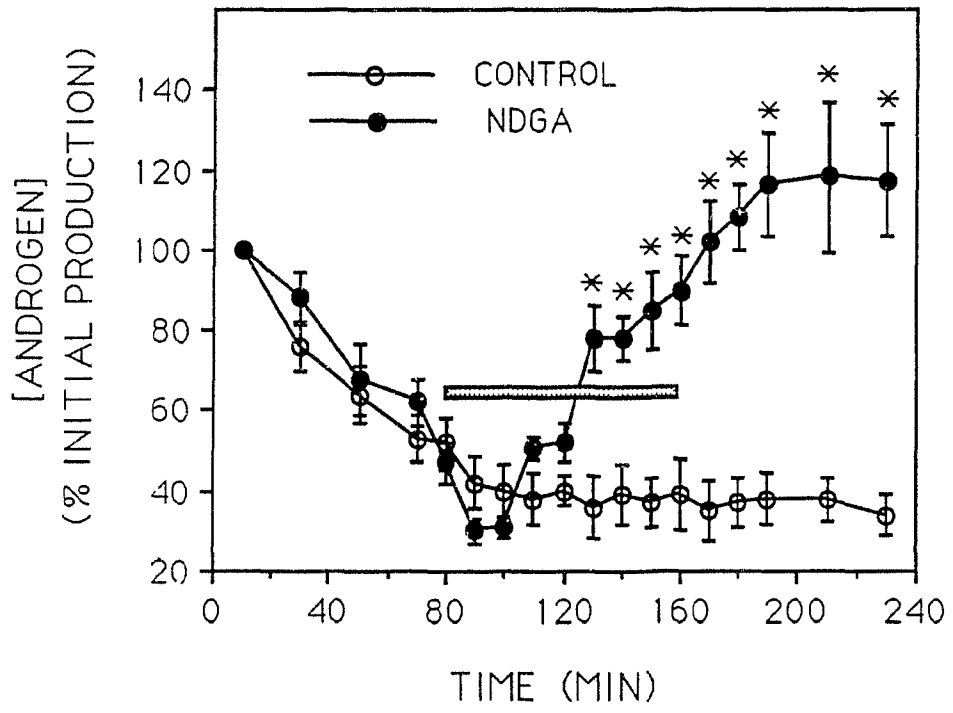


Figure 28. Effect of lipoxygenase inhibition on androgen production by perifused Leydig cells. The cells were perifused with 0 or 50μM NDGA (from 80-160 min, as indicated by the shaded bar). Data are expressed as percentage of androgen levels in the first sample collected. All data are means $\pm$ SEM of 4 separate experiments. * denotes a statistically significant difference ($p \leq 0.05$) relative to the control production.

infused with 100 μM W7 + 50 μM NDGA (Fig. 29) or chronically infused with 10 μM NDGA and subsequently infused with 100 μM W7 (Fig. 30) would suggest that the two agents were acting via the same mechanism as the stimulation of androgen production by the two inhibitors was not additive.

11. The effect of lipoxygenase inhibition on the response of Leydig cells to LH

The effect of lipoxygenase inhibition on the steroidogenic response of Leydig cells to LH (0.3 - 100 ng/ml) was also investigated. As illustrated in Fig. 31, exposure to 20 μM NDGA in static incubations significantly reduced the steroidogenic response to maximal LH stimulation (100 ng/ml) while significantly decreasing the sensitivity of the cells to the lower concentrations of LH concentration (EC_{50} LH = 9.1 ± 1.15 ng/ml, EC_{50} LH/NDGA = 31.1 ± 1.33 ng/ml LH). Transient infusion with 50 μM NDGA also significantly inhibited (Fig. 31, $p \leq 0.02$) the steroidogenic response of perifused Leydig cells exposed to a 5 min pulse of 100 ng/ml LH in a manner analogous to the W7/LH experiments (see Fig. 25). The inhibitory effect of NDGA on the Leydig cell response to the gonadotropin was not as great as that seen in the equivalent experiments using the CaM antagonist which virtually abolished the response to LH (Fig. 25).

Figure 29. The effect of co-exposure to W7 and NDGA on androgen production by perifused Leydig cells. The cells were perifused with media, 50 μM NDGA, 100 μM W7 or 50 μM NDGA + 100 μM W7 (from 80-160 min, as indicated by the shaded bar). All data represent means of a single experiment.

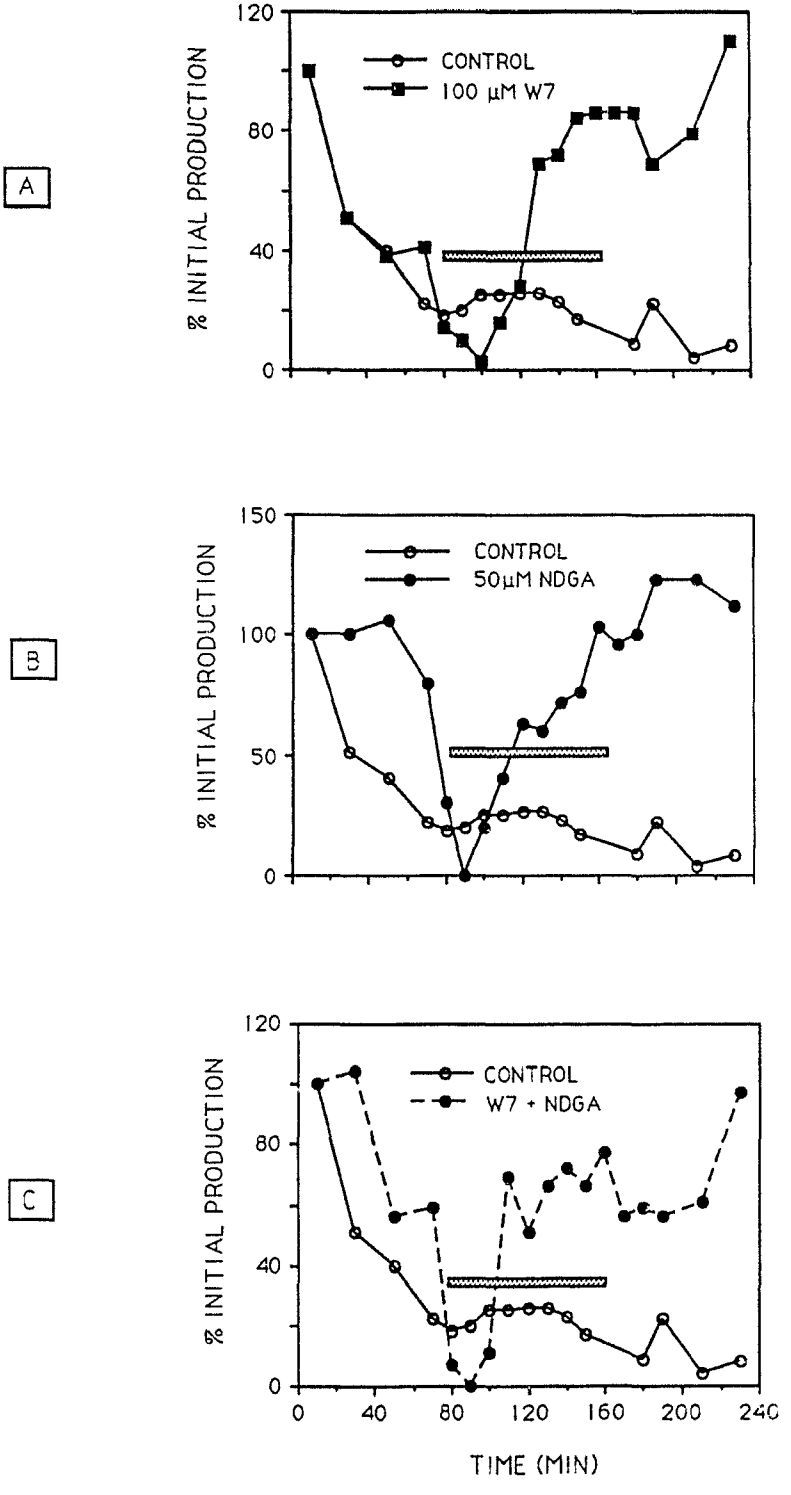

A
120
80
40
0
% INITIAL PRODUCTION
CONTROL
100 µM W7
B
150
100
50
0
% INITIAL PRODUCTION
CONTROL
50µM NDGA
C
120
80
40
0
% INITIAL PRODUCTION
CONTROL
W7 + NDGA
0
40
80
120
160
200
240
TIME (MIN)

Figure 29.

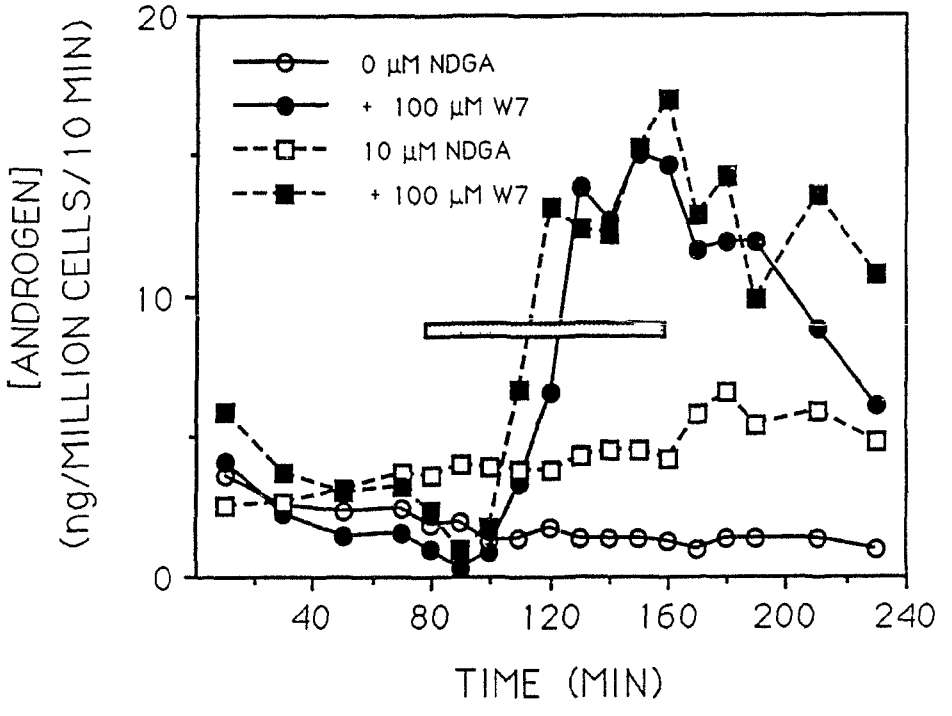


Figure 30. The effect of lipoxygenase inhibition on the steroidogenic response to W7 by perifused Leydig cells. The cells were chronically perifused with 0 or 10 μM NDGA and then perifused with media or 100 μM W7 (from 80-160 min, as indicated by the shaded bar). The data represents means of a representative experiment.

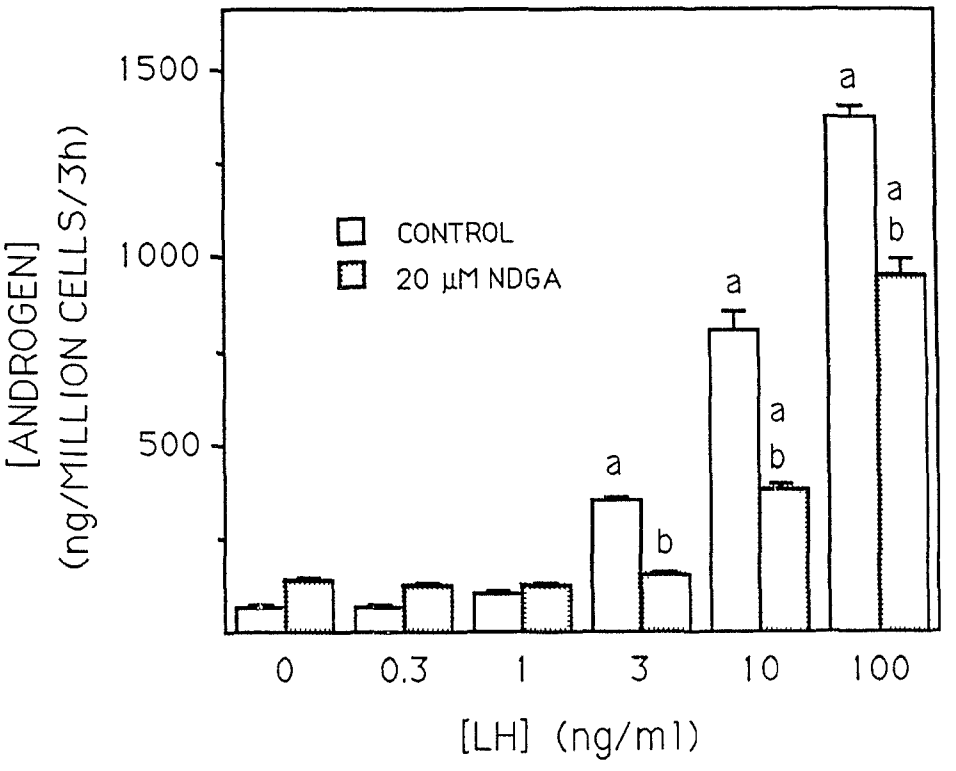


Figure 31. Effect of lipoxygenase inhibition on LH-stimulated androgen production in Leydig cell incubations. Cells were exposed to 0-100 ng/ml LH in the presence or absence of 20 μM NDGA during 3h static incubations as described in Materials and Methods. All data represents means ± SEM of a representative experiment. "a" denotes a statistically significant difference ($p \leq 0.01$) relative to the control production while "b" denotes a statistically significant treatment effect of the NDGA as determined by a Scheffe F-test.

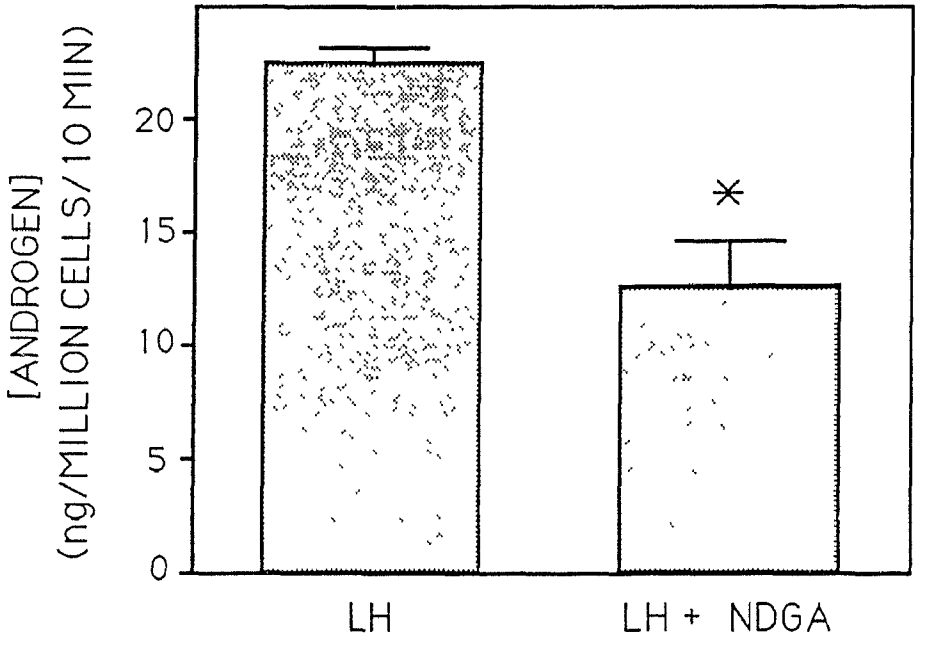


Figure 32. The effect of NDGA on LH-stimulated androgen production by perifused Leydig cells. The cells were perifused with media or 50 μM NDGA (from 80-160 min) and subsequently infused with 100 ng/ml LH for 5 min (beginning at 145 min). Data represents means ± SEM of 3 separate experiments. * denotes a statistically significant difference ($p \leq 0.02$) relative to production from the control chamber.

12. The effect of arachidonic acid on androgen production by incubated Leydig cells

Preliminary experiments showed that arachidonic acid had a biphasic effect on androgen production in Leydig cell incubations (Fig. 33), reminiscent of the effect of both W7 (Fig. 19) and NDGA (Fig. 27). The stimulatory influence of AA confirmed similar findings in the rat Leydig cell (Lin, 1985) although the concentration required to enhance steroidogenic output in the mouse was an order of magnitude above that required in the rat.

C. Discussion

The ubiquitous calcium-binding protein, calmodulin, acts to translate fluctuations in intracellular calcium levels into functional alterations in the activity of cyclic 3',5'-nucleotide phosphodiesterase (PDE) (Cheung, 1970), myosin light chain kinase (Blumenthal and Stull, 1982), phospholipase A_2 (Wong and Cheung, 1979; Nakagawa and Waku, 1988) as well as a number of other enzyme systems (Means, 1988). Hall and coworkers (1981) used the CaM antagonist, TFP, in static incubation studies to demonstrate a stimulatory role for calmodulin in the control of rat Leydig cell function, seemingly by influencing the delivery of cholesterol to the inner mitochondrial membranes.

However, we and others found that incubation of

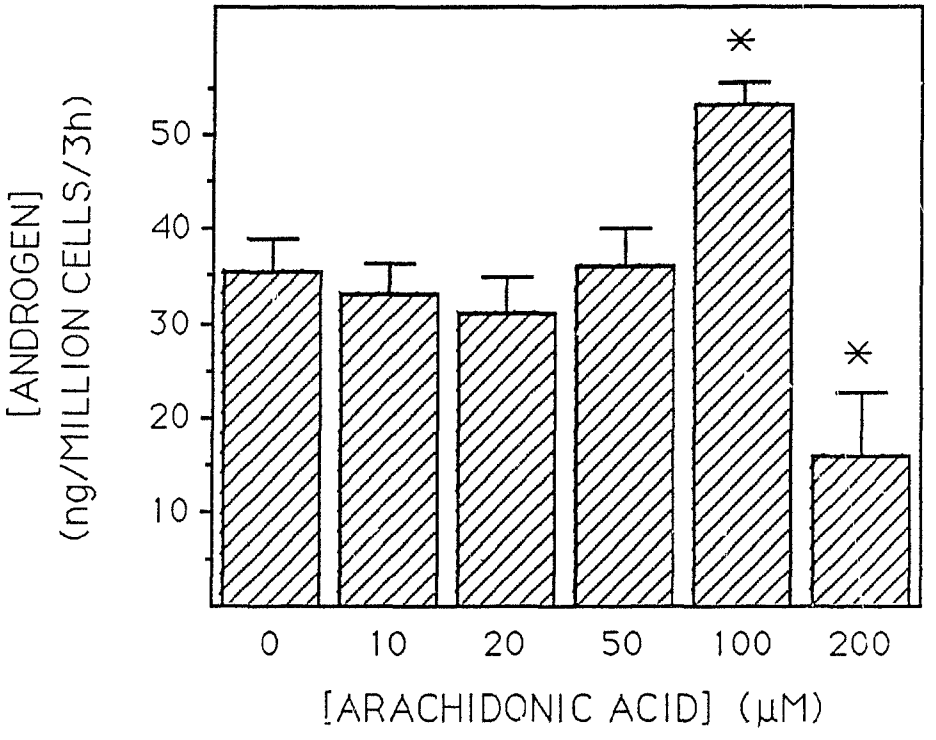


Figure 33. The effect of arachidonic acid on androgen production by Leydig cell incubations. The cells were exposed to 0-200 μM arachidonic acid during 3h static incubations as described in Materials and Methods. All data represents means ± SEM of a representative experiment. * denotes a statistically significant difference ($p \leq 0.05$) relative to the control production.

Leydig cells with CaM antagonists had an interesting but inconsistent biphasic effect on androgen production (Fig. 19; Sullivan and Cooke, 1985) suggesting that CaM may also act in an inhibitory capacity. In order to investigate this possibility, we developed a multichambered cell perifusion system as a more kinetic and therefore, somewhat more informative alternative to static incubations.

In accordance with incubation studies (Hall et al., 1981; Sullivan and Cooke, 1985; Fig. 11), CaM antagonism elicited a biphasic response by the perifused Leydig cells consisting of an initial inhibition followed by a stimulation of androgen production (Figs. 21A, 21B and 22). However, the inhibitory effect was not CaM-specific as exposure to equivalent levels of W5 (100 μM), the dechlorinated analogue of W7 (Tanaka et al., 1982), was equally effective in inhibiting steroidogenesis (Fig. 23). Dose response studies further revealed that even very low concentrations of W7 were sufficient to transiently inhibit steroidogenic function (Fig. 22) suggesting that the effect was not mediated through CaM antagonism. The inability of exogenous cholesterol to abolish the inhibitory response to W7 (Table II) provided convincing evidence that the disruption of cholesterol delivery to the P450 enzymes was not involved. The nonspecificity of the inhibitory effect was very unusual in that low levels of W5 were also effective in reducing androgen release (Section 4). Studies which demonstrated a calmodulin-independent activity for W7

almost universally noted that W5 was ineffective in eliciting a similar response (Hikada et al., 1981; Tanaka et al., 1982; Watanabe et al., 1986). This is not surprising as the antagonistic capabilities of W7 was thought to derive from its hydrophobicity (Tanaka et al., 1982). The lack of the chlorine moiety in W5 greatly reduces that hydrophobicity and its potency as an antagonist (Tanaka et al., 1982). Therefore, the ability of W7/W5 to inhibit androgen production must reside in some chemical characteristic other than the ability to interact with hydrophobic sequences.

There are a number of studies which showed that high levels of W7 nonspecifically inhibited a number of intracellular enzyme systems including protein kinase C (Tanaka et al., 1982; MacNeil et al., 1988), transglutaminase (MacNeil et al., 1988), PLA_2 (Watanabe et al., 1986) and calpain 1 (Brumley and Wallace, 1989). However, taking into account the dilution characteristics of the perifusion system (Fig. 20), the minimally effective concentration of W7 required to stimulate androgen production ($\approx$ 30 μM, Fig 22) is within the effective range of the IC_{50} of W7 for calmodulin-dependent activities ($\approx$30μM) (Hikada et al., 1981; MacNeil et al., 1988) and well below the concentrations which antagonized the interaction of phospholipid cofactors with PLA_2 (IC_{50} $\approx$ 200 μM) (Watanabe et al., 1986) and protein kinase C ($\approx$ 340 μM) (MacNeil et al., 1988).

The reduction of LH responsiveness arising from W7 exposure (Fig. 25) appears to be CaM-specific, however, as exposure to identical treatment levels of W5 (100μM) had no significant effect on the steroidogenic response to the gonadotropin (Fig. 25). The attenuation of Leydig cell responsiveness to LH by CaM antagonists has been noted in earlier studies (Hall et al., 1981; Sullivan and Cooke, 1985) and has been explained on the basis of reduced cholesterol delivery to the inner mitochondrial membranes and the inhibition of $P450_{SCC}$ (Hall et al., 1981). The possibility that exogenous cholesterol could restore gonadotropin responsiveness was not investigated.

Aside from the initial inhibition of steroidogenesis, the stimulation of androgen production by W7 supported the presence of a novel CaM-sensitive inhibitory system within the mouse Leydig cell (Figs. 21A and 22). This stimulatory effect was dose-dependent (Fig. 22) and CaM-specific as exposure to 100μM W5 was not successful in stimulating steroidogenesis. (Figs. 23). This confirmed our suspicions spurred by the incubation findings from this and other laboratories (Fig. 19; Sullivan and Cooke, 1985) in which exposure to CaM antagonists appeared capable of both stimulating and inhibiting androgen production.

There are several possible explanations for a W7-mediated enhancement of androgen production, the first being related to the aforementioned nonspecific W7-mediated inhibition of androgen production. There could be a pooling

of cholesterol during the transitory inhibition following exposure to 100 μM W7. If this was the case, then cholesterol could suddenly become available to the $P450_{scc}$ and an elevation in androgen production could ensue. However, the transition from inhibition of androgen production to a stimulatory response seen with 100 μM W7 occurred before the inhibitory effects of the lower concentration of the CaM antagonist had subsided (Fig. 21B). In other words, the initial CaM-independent inhibitory response to W7 was still underway even as the stimulation mediated by CaM antagonism commenced. In addition, exposure to lower concentrations of W7 or to 100 μM W5 resulted in a virtually identical inhibitory response without the ensuing stimulatory effect (Figs. 21 and 22). This would certainly contraindicate any causal relationship between the initial inhibitory effect of W7 and the subsequent stimulation of androgen production.

Another possible explanation for the stimulation in androgen production by W7 was sparing of cAMP and/or cGMP through inhibition of a calmodulin-dependent cyclic 3',5'-nucleotide phosphodiesterase (Levin and Weiss, 1977; Hikada et al., 1984). While direct measurement of cyclic nucleotide levels in the perifusion sample was inconclusive (Table III and IV), tonic inhibition of the PDE enzyme with MIX concurrent with W7 exposure had no effect on the stimulation of androgen production by the CaM antagonist (Fig. 24). If the increase in androgen production resulted

from reduced hydrolysis of cyclic nucleotides, then PDE inhibition prior to W7 exposure should have attenuated the stimulatory response. If the stimulation of androgen production was mediated through the generation of new cAMP/cGMP, then PDE inhibition should potentiate the effect. The fact that neither of these scenarios came to pass strongly suggests that cyclic nucleotides do not mediate this phenomenon.

Of course, there is evidence of cyclic nucleotide-independent messenger systems in the Leydig cell including the eicosanoid cascade (Lin, 1985; Sullivan and Cooke, 1986). Recently, Abasyasekara et al. (1990) demonstrated that treatment with exogenous phospholipase A_2 (PLA_2), the rate-limiting enzyme in the eicosanoid cascade (Wolfe, 1982) increased androgen synthesis in rat Leydig cells without any parallel increase in cAMP accumulation. This supported earlier findings by Lin (1985) in which exogenous arachidonic acid stimulated testosterone accumulation in rat Leydig cell incubations. A number of enzymes within the eicosanoid cascade are calcium-dependent including phosphlipase A_2 (Pickett et al., 1977) and 5-lipoxygenase (Jakshik et al., 1980; Rouzer et al., 1985). There is a possibility that some of these calcium-sensitive enzymes may also require calmodulin for maximal activity. <u>In vitro</u> studies have consistently found that optimal activation of the purified PLA_2 or lipoxygenase required supraphysiological levels of Ca^{2+} ($EC_{50} \approx 0.1\text{-}1.0$ mM)

(Jakshik et al., 1980; Rouzer et al., 1985; Shimizu, 1988). This prompted Shimizu (1988) to theorize that a factor may be present in situ which acts as an intermediary to more efficiently couple the lipoxygenase enzyme with the calcium ion. Calmodulin, as a calcium binding protein (Means, 1988), could act in this capacity as appears to be the case for the PLA_2 enzyme (Wong and Cheung, 1979; Craven and DeRubertis, 1985; Nakagawa and Waku, 1988). Indeed, findings that the stimulatory W7 effect could be abolished by prior inhibition of arachidonic acid release with 20 μM mepacrine (Table V) could be interpreted as an indication of an inhibitory calmodulin-dependent PLA_2 system. If this was the case, however, transient exposure of the Leydig cells to mepacrine in a manner analogous to the W7 treatments could be expected to mimic the effect of the CaM antagonist. In fact, a 20 min pulse of 20 μM mepacrine had no stimulatory effect on androgen production by perifused Leydig cells (Table VI). This is in agreement with the aforementioned studies in the rat which revealed that exogenous arachidonic acid or PLA_2 stimulated, rather than inhibited Leydig cell function (Lin, 1985; Abasyasekara et al., 1990) while PLA_2 antagonism has also been shown to inhibit LH-stimulated Leydig cell steroidogenesis (Abasyasekara et al., 1990).

The effect of PLA_2 inhibition on the stimulatory effect of W7 could, however, be a consequence of the depletion of AA as a substrate for a calmodulin-sensitive enzyme(s) farther downstream within the eicosanoid pathway.

Although products of the cyclooxygenase pathway do not appear to play a large role in Leydig cell function (Sullivan and Cooke, 1985; Fig. 26), exposure to NDGA at levels appropriate for the specific inhibition of lipoxygenase (Tappel et al., 1953) stimulated basal androgen production in short term incubations of mouse and rat Leydig cells (Fig. 27; Dix et al., 1984; Lin, 1985; Sullivan and Cooke, 1985). Further studies revealed that transient exposure to NDGA had a biphasic effect on androgen production by perifused Leydig cells (Fig. 28). This response was reminiscent of that elicited with higher concentrations of W7 (Fig. 21 and 22) although the maximal stimulatory response to NDGA (≈ 210 min) occurred somewhat later than that with W7 (≈ 160 min, Fig 21A). The difference in the response time was not surprising in that though both drugs may well inhibit the lipoxygenase enzyme, the means by which this is accomplished may differ. NDGA is a phenolic antioxidant which inhibits the lipoxygenase activity by modifying sulfhydryl moieties on the enzyme (Needleman et al., 1986) while W7 would conceivably interfere with the requirement of the enzyme system for Ca^{2+}. Preliminary findings that neither co-infusion with 50 μM NDGA nor chronic lipoxygenase inhibition significantly enhanced the steroidogenic response to the CaM antagonist (Figs. 29 and 30) also suggested that the two inhibitors were both stimulating androgen production via the same mechanism of action.

The delay in maximal stimulation of androgen production by NDGA relative to that seen with W7 may, in part, explain why the lipoxygenase inhibitor, while effective in inhibiting the maximal response to LH (at ≈ 160 min; Fig. 32), was not as effective as 100 μM W7 (Fig. 25). These experiments were designed such that direct comparisons could be made between the inhibitory effects of NDGA and W7 on the steroidogenic response to LH. However, if the experiments had been designed such that the maximal LH response coincided with the time of maximal NDGA-mediated stimulation of androgen production (at ≈ 210 min) as were the W7 experiments (at ≈ 160 min), then the inhibitory influence of NDGA may have been more profound. In addition, it is unlikely that the inhibitory effect of W7 on the steroidogenic response to LH was mediated exclusively by the inhibition of lipoxygenase function. For example, the inhibition of a CaM-sensitive cholesterol delivery system (Hall et al., 1981) or other calmodulin-mediated events could also be involved. Preliminary incubation studies also showed that although basal androgen production was stimulated by NDGA (Fig. 27), LH-stimulated steroidogenic response of Leydig cells was inhibited (Fig. 31). Lipoxygenase inhibition significantly increased the threshold of the cell to activation by LH while reducing the maximal response to the gonadotropin (Fig. 31). This latter finding confirmed some of the earlier studies in the rat in which NDGA inhibited both androgen and cAMP production in

response to maximally stimulatory levels of LH (Dix et al., 1984).

These findings provide indirect evidence that W7 may be increasing androgen production in the mouse Leydig cell via the suppression of a tonically inhibitory, CaM-sensitive lipoxygenase enzyme. Inhibition of steroidogenic function by the lipoxygenase system could be mediated via two different mechanisms: directly by synthesis of an inhibitory factor or indirectly, by inhibition of a stimulatory influence.

In the first scenario, the lipoxygenase enzyme is responsible for the synthesis of an actively inhibitory eicosanoid factor. This would seem unlikely as Sullivan and Cooke (1988) found that exposure to the 5-lipoxygenase product, 5-hydroperoxyeicosatetraenoic acid stimulated androgen production by rat Leydig cells although the product of another lipoxygenase could conceivably act in this capacity. The fact that indirect inhibition of lipoxygenase activity by interference with AA synthesis did not mimic the stimulatory effect of W7/NDGA (Table VI) provides the more compelling argument against the lipoxygenase-mediated synthesis of an inhibitory factor.

In the second scenario, the lipoxygenase enzyme attenuates the influence of an unknown stimulatory factor. Inhibition of the enzyme by W7/NDGA would induce an increase in the intracellular levels of this factor and androgen production would be enhanced. A strong candidate for this putative stimulatory vector is arachidonic acid. Lin (1985)

demonstrated that stimulation of androgen production in the rat Leydig cell by AA was potentiated by inhibition of the cyclooxygenase and lipoxygenase enzymes suggesting that arachidonic acid mediated a stimulatory function in the Leydig cell. The AA-mediated activation of androgen production was also demonstrated in some preliminary incubation studies with the mouse Leydig cell (Fig. 33). Although hardly conclusive evidence, the biphasic characteristics of the steroidogenic response to W7 (Fig. 19), NDGA (Fig. 27) and AA (Fig. 33) in incubations is suggestive of a common mechanism of action.

The mechanism by which arachidonic acid could stimulate Leydig cell steroidogenic function has not been clearly elucidated. It is conceivable that the AA could be utilized as a substrate for an epoxygenase cytochrome P450 enzyme system (Needleman et al., 1986) as concentrations of NDGA up to 50 μM were ineffective in inhibiting this system in the liver microsomes (Turk et al., 1990). Although inhibitors for this enzyme system exist, they are very nonspecific and tend to inhibit all P450 enzyme systems (Luini and Axelrod, 1985). This would severely limit the usefulness of these inhibitors in the investigation of the role of the epoxygenase pathway in the Leydig cell and other steroidogenic tissues.

Free AA has been shown to activate phospholipase C (Zeiller and Handwerger, 1985) and to trigger a rise in cytosolic calcium levels independent of inositol

triphosphate release (Wolfe et al., 1986). Activation of Ca^{2+}/phospholipid-dependent protein kinase by arachidonic acid has been reported (McPhail et al., 1984) and has been shown to occur in rat Leydig cells (Lin, 1985). Activation of PK-C by a W7/NDGA-mediated accumulation AA could also play a role in the inhibition of the steroidogenic response to LH (Themmens et al., 1986; Mukhopadhyay et al., 1986).

VI. GENERAL DISCUSSION

Many of the attempts to isolate and study a particular second messenger cascade fail to account for potential interaction with other second messenger systems. Indeed, this tendency to categorize these messenger systems as insulated linear organizations probably more accurately reflects on the investigative limitations than on the nature of cellular organization. It is apparent from the findings in this study and from reviewing the literature that the intracellular control of cellular activities via second messengers is both interactive and interdependent. As more information emerges regarding the complexity of these interactions, it becomes apparent that these separate "messenger cascades" are actually components of a single "messenger network". Within this network, different cascades may predominate during certain circumstances (ie. LH-mediated increase in cAMP synthesis) and in doing so, overshadow a whole host of more subtle responses which act to fine-tune the cellular response. For example, the influx of Ca^{2+} which accompanies LH-mediated increases in cAMP synthesis (Sullivan and Cooke, 1986) can activate PK-C which will, in turn, desensitize the LH receptor/adenylate cyclase complex to further stimulation (Themmens et al., 1986, Innoue and Rebois, 1989). Phosphorylation and activation of cyclic nucleotide PDE by PK-A (Corbin et al.,

1988) is another example of interactive communications but within a single messenger cascade. Not all second messenger interactions are adversarial; some may be cooperative as illustrated by the synergistic enhancement of androgen production by cAMP and cGMP (Study 2). Be it positive or negative, these ultrashort feedback loops provide precise mechanisms through which cellular responses can be strictly controlled.

A. The functional importance of PK-A isoforms in mouse Leydig cell steroidogenesis

Though both of the predominant isoforms of cAMP-dependent kinase have been isolated in normal and tumour Leydig cells (Cooke et al., 1976; Pereira et al., 1987), the functional importance of the individual isoforms had not been previously assessed. Findings in this study suggested that both type 1 and 2 isoenzymes were functionally active in the control of testicular steroidogenesis (Figs. 8, 10 and 11). The difference in the potential of each isoenzyme in regulating the synthesis of androgens (Fig. 8) raises a number of interesting questions about compartmentalization within a single messenger system. Compartmentalization of the different PK-A isoenzymes may involve functional as well as distributional disparities within the cell. Classically, discussion about compartmentalization revolved around

differences in distribution within the cell (ie. cytoplasmic vs membrane-bound, bound vs free, et cetera) and how this influenced the functional capabilities of that component (Moger, in press). This relationship is certainly valid but may underestimate the complexity of the situation. For example, differential sensitivity to autophosphorylation between the two major isoenzymes of PK-A may well reflect a functional disparity which is independent of cellular distribution. Findings in Leydig tumour cells suggest that the T2 PK-A is simply less active than the T1 enzyme (Pereira et al., 1987) and this may explain the somewhat diminished abilities of that isoenzyme in activating steroidogenesis in the normal Leydig cell (Figs. 5, 7 and 8). Type 2 PK-A has been shown to exist primarily in a phosphorylated and therefore, hypersensitive state (Rangel-Aldao et al., 1979). The heightened affinity of the phosphorylated type 2 isoform for cAMP may allow for its activation by basal levels of cAMP facilitating the synthesis of androgens in the absence of steroidogenic activators. The ability of the site/type-selective cAMP analogues to activate the T2 PK-A isoform would not only be influenced by the levels of endogenously generated cAMP but also by the availability of intact (R_2C_2) enzyme. Therefore, the relatively smaller synergistic interaction noted with co-exposure to LH/cAMP and the type 2 analogues may, at least in part, result from the reduced availability

of intact T2 holoenzyme. An assay of phosphotransferase activity in the normal mouse Leydig cell akin to that done in the rat and tumour Leydig cell (Cooke et al., 1976; Pereira et al., 1987) would be helpful in gaining some insight into the quantitative activities of the PK-A isoenzymes. If there is some functional compartmentalization between the PK-A isoforms, whether based on cellular distribution or functional characteristics, then there may also be some difference in substrate specificity.

The attenuated synergistic interaction between the cAMP analogues and forskolin suggests that the diterpene may have cAMP-independent activities (as discussed in Study 1). Some of these activities have been characterized in the rat Leydig cell, including the inhibition of glucose transport across the cell membrane (Amrolia et al., 1988) by forskolin levels which were almost ten-fold greater than the concentration used in these experiments. As forskolin is utilized extensively in second messenger studies (Seamon et al., 1981), it is important to more fully characterize any potential nonspecific actions of the drug. This was begun with preliminary studies using 1,9-dideoxyforskolin, a naturally occurring forskolin analogue which does not activate adenylate cyclase (Laurenza et al., 1989). Previous studies found inhibitory effects of forskolin at very low (pM) or fairly high (5-10 μM) concentrations (Dufau et al., 1987; Amrolia et al., 1988; Laurenza et al., 1989). This

study provided preliminary evidence that concentrations of forskolin which were commonly utilized experimentally (1 μM) (Moger and Anakwe, 1983; Peirera et al., 1987; Nikula and Huhtaniemi, 1989) elicited inhibitory effects independent of the cAMP-mediated stimulation of androgen production.

It is apparent that a great deal remains to be done before the intricacies of cAMP-mediated activation of cellular function will be fully understood.

B. Interaction between cAMP and cGMP second messengers

The "Yin-Yang" hypothesis proposed by Goldberg and coworkers (1975) was based on experimental findings in which cellular activities were either stimulated by cAMP and inhibited by cGMP or were promoted by cGMP and inhibited by cAMP. The acceptance of this hypothesis was fairly widespread and still influences scientific thinking to this day.

However, like any good rule there are always exceptions. There was evidence that in certain cell types, such as the mouse Leydig cell, both cAMP and cGMP could drive the same response in the same direction. Findings in this study indicated that two hormonal factors which enhanced androgen production via two different cyclic nucleotide second messenger systems (LH and ANF), interacted synergistically to activate steroidogenesis in the murine

Leydig cell (Fig. 12). It was not surprising, therefore, that cAMP and cGMP did not just act in parallel but also interacted cooperatively to increase androgen output (Figs. 13 and 14). The fact that this positive interaction occurred only with low stimulatory levels of LH/cAMP and ANF/cGMP suggested that it may be of physiological relevance in the hormonal control of testicular steroid production. Although the ANF prohormone and its mRNA has been recently isolated in the rat testis (Vollmar et al., 1990), the role of ANF in the endocrine or paracrine control of Leydig cell function in vivo has not, as of yet, been conclusively established in the rodent or human testes.

The most obvious question which remains to be answered is how this interaction is mediated (Fig. 34). Although the cGMP-dependent protein kinase may directly influence the phosphorylative state and therefore, the activity of the cAMP-dependent kinases in an intermolecular fashion (Gealhen and Krebs, 1980; Gealhen et al., 1981), any interaction of cAMP and cGMP at the level of a common phosphotransferase would probably involve the enhancement of intramolecular autophosphorylation (deJong and Rosen, 1977; Lincoln and Corbin, 1983; see Fig. 31). Measuring the extent of regulatory subunit phosphorylation upon co-exposure to LH/cAMP and ANF/cGMP would provide a definitive assessment of the physiological relevance of this level of interaction in the mouse Leydig cell. An attempt should also be made to

Fig. 34. Putative interaction between the cyclic nucleotide second messenger systems in the mouse Leydig cell. The cooperative interaction between the LH/cAMP and ANF/cGMP systems and the possible mechanisms through which that interaction may elicit the synergistic increase in androgen production.

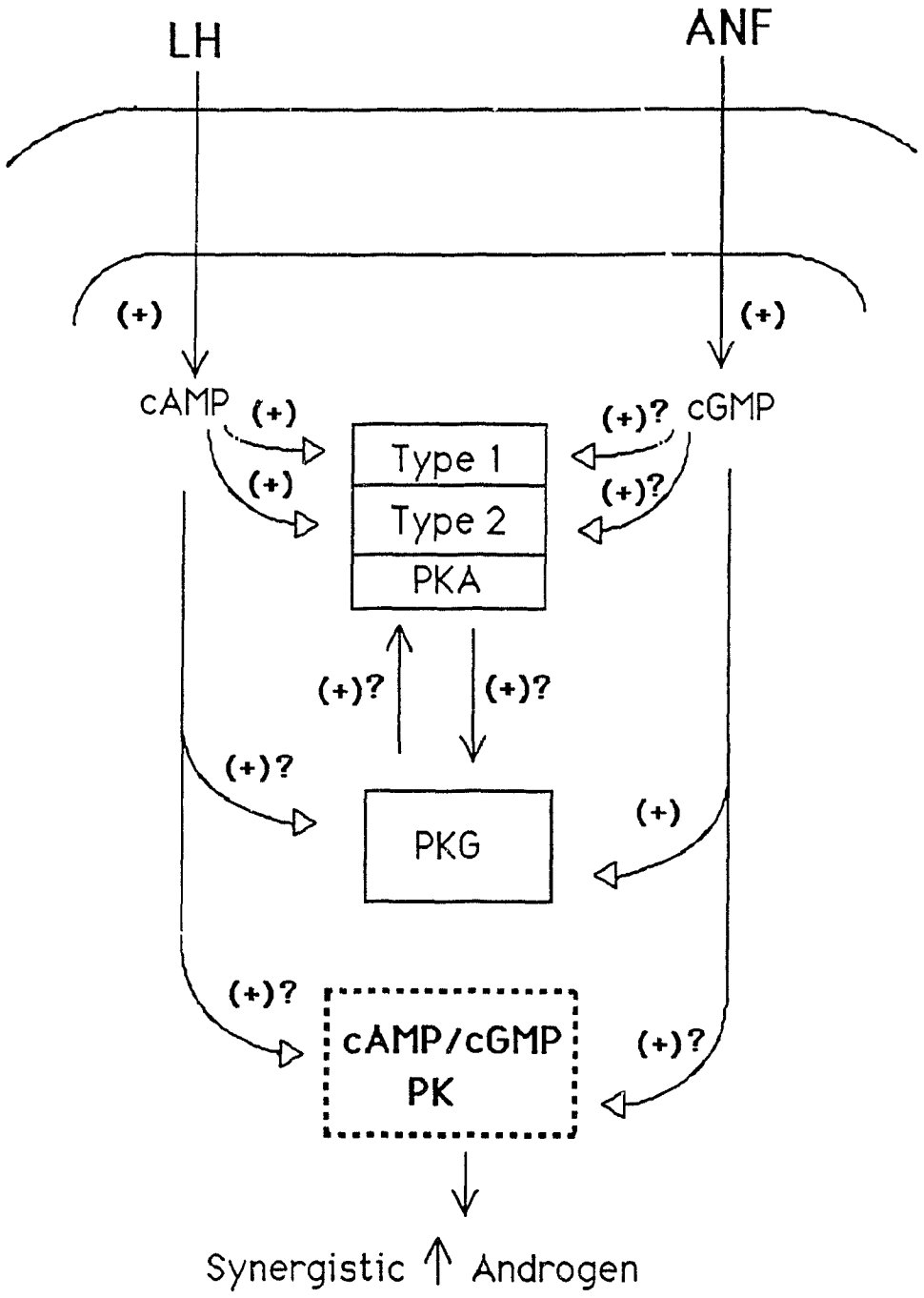

LH
ANF
(+)
(+)
cAMP
(+)
(+)?
cGMP
(+)
(+)?
Type 1
Type 2
PKA
(+)?
(+)?
(+)?
PKG
(+)
(+)?
cAMP/cGMP
PK
(+)?
Synergistic
Androgen

Figure 34.

isolate a cAMP/cGMP-dependent protein kinase (Vardanis, 1980) from the Leydig cell and other cell types in which the two cyclic nucleotides have been shown to act cooperatively (Ho et al., 1988; Furman and Tanaka, 1989). Although cGMP and the cAMP analogues seemingly interact positively beyond the site of synthesis, ANF also desensitizes somewhat the Leydig cell to highly stimulatory levels of gonadotropins (Pandey et al., 1985, 1986; Fig. 12). The possibility that this attenuation of response may be mediated through stimulation of cAMP hydrolysis by type II PDE has yet to be addressed.

C. An inhibitory, calmodulin-sensitive lipoxygenase system in the mouse Leydig cell

Another example of second messenger interdependence relates to the W7 studies in this thesis and is schematically represented in Figure 35. Inhibition of CaM with the antagonist W7 elicited an increase in androgen secretion by perifused Leydig cells (Figs. 18, 19 and 20) suggesting that a tonically inhibitory CaM-sensitive process exists within this cell type. Inhibition of arachidonic acid synthesis with mepacrine abolished the stimulatory response to W7 (Table III) while the reduction AA utilization by the lipoxygenase enzyme farther downstream in the eicosanoid cascade mimicked the response to W7 (Figs. 24 and 25). These

Fig. 35. Inhibition of a Ca^{2+}/calmodulin-sensitive lipoxygenase enzyme in the mouse Leydig cell. The putative mechanisms through which the CaM antagonist, W7, stimulates androgen production in the mouse Leydig cell.

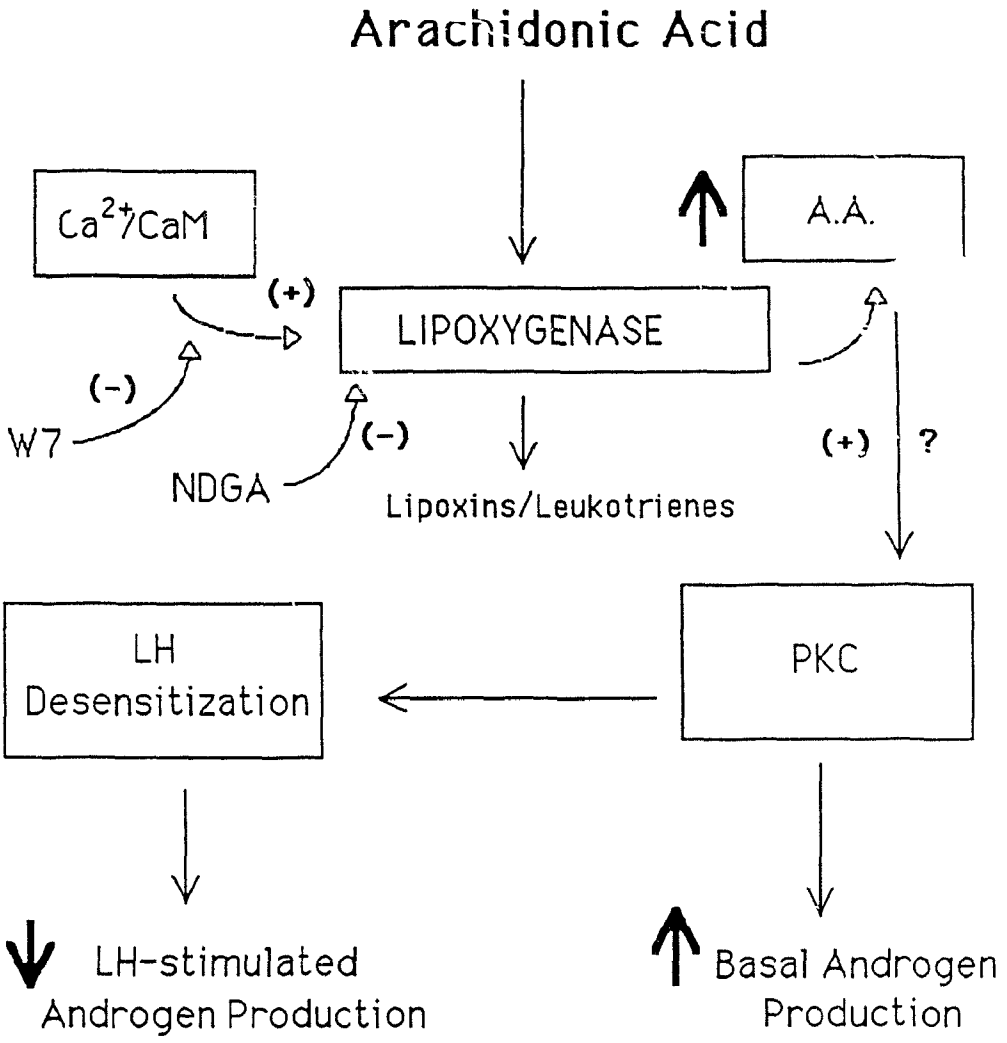

Arachidonic Acid
Ca^{2+}/CaM
A.A.
(+)
LIPOXYGENASE
(-)
W7
(-)
NDGA
Lipoxins/Leukotrienes
(+)
?
LH
Desensitization
PKC
LH-stimulated
Androgen Production
Basal Androgen
Production

Figure 35.

data suggest that arachidonic acid accumulation due to lipoxygenase inhibition by W7/NDGA was responsible for the increase in androgen production. Lin (1985) originally established that AA could stimulate androgen production by the rat Leydig cell and preliminary incubation studies (Fig. 30) suggested a similar influence in the mouse Leydig cell. Lin (1985) also established that AA could both activate protein kinase C and increase its sensitivity to both ionic calcium and phospholipids. The possibility that Ca^{2+}/CaM inhibits Leydig cell function via enhancement of lipoxygenase-mediated AA utilization suggests the potential for an interesting negative feedback loop. For example, the stimulation of rat Leydig cell steroidogenic function by GnRH is temporally associated with a rise in intracellular calcium concentrations (Sullivan and Cooke, 1986) and appears to be mediated via protein kinase C activation (Lin, 1985). The steroidogenic response to GnRH was potentiated by lipoxygenase inhibition or by exogenous arachidonic acid (Lin, 1985). A rise in free calcium levels within the cell following exposure to GnRH would elicit conformational changes in CaM thereby facilitating its binding to the lipoxygenase enzyme. The lipoxygenase enzyme would then utilize AA at a faster rate and reducing its availability as a PK-C cofactor, attenuating the steroidogenic response to GnRH. In this manner, the response to the stimulatory factor (GnRH) would be self-limiting.

Although the W7 studies contained in this thesis certainly suggest the presence of a CaM-sensitive lipoxygenase enzyme in the Leydig cell, they do not provide definitive proof. Studies which use pharmacological agents as tools to examine physiology simply act as catalysts for more intensive studies. To firmly establish the presence of a CaM-sensitive lipoxygenase system, more specific biochemical and molecular approaches must be instigated.

D. Summary and conclusions

The general objective of this study as previously stated was to more fully characterize the role of various second messenger networks in the mouse Leydig cell and to assess the potential for interaction between these intracellular factors in the regulation of testicular androgen production. The findings of the studies in this thesis, which are summarized in the following paragraphs, provide some new insights on the complexity and interdependency of these second messengers sytems.

Analogues of cyclic adenosine 3',5'-monophosphate (cAMP) selective for either binding site (S1 and S2) on the regulatory subunits of cAMP-dependent protein kinases (PK-A) were used to assess the role of type 1 (T1) and type 2 (T2) PK-A. Both major iscenzymes of cAMP-dependent protein kinase were present and capable of activating androgen production

in the murine Leydig cell though the type 1 isoform was functionally dominant. There was some evidence that forskolin may have cAMP-independent activities which inhibited the ability of cAMP analogues to synergistically activate steroidogenesis.

Although atrial natriuretic factor (ANF) stimulates T output via cyclic guanosine 3',5'-monophosphate (cGMP), coexposure of Leydig cells to ANF and LH or ANF/cGMP and the same cAMP analogues resulted in a synergistic increase in T production suggesting cyclic nucleotide interaction mediates a cooperative hormonal control of the mouse Leydig cell in vivo.

The role of calmodulin (CaM) in Leydig cell function was assessed using a CaM antagonist, N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W7) and a multichambered cell perifusion system. Transient exposure to W7 elicited an initial nonspecific inhibition of androgen production followed by a CaM-dependent increase in steroidogenesis suggesting the presence of a novel inhibitory CaM-sensitive process in the LC. However, co-exposure to highly stimulatory levels of LH revealed that W7 elicited a CaM-specific inhibition in gonadotropin responsiveness. The stimulatory response to W7 was not attenuated by chronic PDE inhibition indicating that the inhibition of a CaM-dependent PDE was not a factor in the response. The W7-mediated increase in androgen production was abolished, however, with

chronic exposure to mepacrine, a phospholipase A_2 inhibitor and mimicked with transient exposure to a lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). NDGA also attenuated the steroidogenic response to LH both in incubations and perifusions. The stimulatory response to W7 and NDGA co-infusion was not additive suggesting a common mechanism of action, ie. the inhibition of arachidonic acid utilization by a CaM-sensitive lipoxygenase enzyme. Preliminary incubation studies supported this possibility, demonstrating that exogenous AA enhanced androgen production.

It is concluded that interactions between second messenger systems play an important role in the control of androgen production by murine Leydig cells.

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