REGULATION OF STEROIDOGENESIS IN SUBCLONES OF THE
MA-10 MOUSE LEYDIG TUMOR CELL LINE

by

MICHAEL WAYNE KILGORE, B.S., M.S.

A DISSERTATION

IN

BIOCHEMISTRY

Submitted to the Graduate Faculty of
Texas Tech University Health Sciences Center
in Partial Fulfillment of
the Requirements for
the Degree of

DOCTOR OF PHILOSOPHY

Approved

Chairperson of the Committee

Accepted

Dean of the Graduate School

May, 1990

21
T2
1990
No. 150
C-1-2

ACKNOWLEDGMENTS

First and foremost I would like to thank my wife, Malissa. Hers has been the voice of reason and understanding when I needed them most. Her love, insight and advice have carried me through the difficult times and made the good times better. I could not have made it without you. We've come a long way and there is yet a long trek ahead, but with you it will seem all too brief.

To my family I owe much of who I am and what I've accomplished. I would like to thank my parents who have supported me in my failures and my successes and were always there when I needed them most. I am indebted to my brother Billy who has been and always will be my best friend-we are of a feather. I would also like to thank my brother John who has shown me the power of perseverance and dedication.

For advice, support and assistance I would like to thank all the members of my committee, Drs. James Hutson, Charles Garner, Kenneth Barker, Ram Chaudhary and Charles Faust. I would like to express my appreciation for the friendship and technical assistance of Debra DeHaven Alberts. I also acknowledge the support of grants HD-00685 and RR-03489 awarded to Dr. Douglas Stocco and two years of support from the Reproductive Training Grant HD-17481.

To Dr. George Stewart, a mentor and friend, I thank you for your help and your patience.

Finally, I would like to express my greatest appreciation for Dr. Douglas Stocco who has shown me what it will take to succeed. Your insight and direction have helped to shape me as a scientist and a person. The lessons you have taught me will be with me throughout my career. In my life I hope to teach a student of mine what you have taught me. I am forever in your debt.

CONTENTS

ACKNOWLEDGMENTS	ii
ABSTRACT	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
I. INTRODUCTION	1
Historical	1
The Testis	2
Regulatory Mechanisms	3
Biochemical Mechanisms of LH Action	4
II. MATERIAL AND METHODS	10
Chemicals	10
Cell Culture	11
Cell Cloning	12
RIA	13
cAMP Binding Assay	14
3 β -Hydroxysteroid Dehydrogenase Assay	14
Cytochrome C Oxidase Assay	15
Cholesterol Side-Chain Cleavage Assay	16
Protein Kinase A Activity	17
Isolation of Mitochondria	17
Radiolabelling of Cells	19
Two-Dimensional Gel Electrophoresis	19
Quantitation of Proteins on Gels	20
Statistical Analysis	21
III. RESULTS	22
Mycoplasma	22
Production of Steroid	22
Cholesterol Side-Chain Cleavage Activity	31
cAMP Production	34
Protein Kinase A Activity	39
SCP ₂ Effects on Isolated Mitochondria	39
Effects of Calcium Inhibitors on Whole Cells	41
Whole Cell Treatment effects on Mitochondria	44
Mitochondrial Reconstruction Experiments	46
Mitochondrial Protein Profiles	51
IV. DISCUSSION	59
BIBLIOGRAPHY	68

ABSTRACT

Cloned cell lines have proven to be useful models in understanding the regulation of endocrine cells and steroid hormone biosynthesis. In an earlier report on the use of cell clones (1) we reported the isolation and initial characterization of a subclone of the MA-10 mouse Leydig tumor cell line (MA-10 LP) which secreted less than 10% of the steroid synthesized by the parent when stimulated with hCG or Bt₂cAMP while basal levels of steroid production were indistinguishable between the two cell populations. In both clones, hCG resulted in the accumulation of comparable amounts of cAMP up to 1 h in the presence of a phosphodiesterase inhibitor or at 30 min in the absence of any inhibitors. Measurement of cholesterol side-chain cleavage (CSCC) activity, the rate limiting enzymatic step in steroidogenesis, using two separate methods demonstrated no difference in the activity of this enzyme complex. In a subsequent report (2) we have shown that addition of exogenous sterol carrier protein 2 (SCP₂) to isolated mitochondria was not able to overcome the deficient steroid response of MA-10 LP. We have also demonstrated that hCG stimulated cellular events which activate steroid production by subsequently isolated mitochondria. Like whole cells this process requires ongoing protein synthesis, the release of intracellular calcium and is mediated through the calcium-calmodulin complex. Additionally, mitochondrial sonicates from hCG stimulated parent cells were able to stimulate steroid production by intact mitochondria isolated from unstimulated parent cells, whereas sonicates from similarly treated MA-10 LP had no effect on steroid production in these mitochondria. This observation does not appear to be due to the presence of inhibitory factors in the mitochondrial sonicates of MA-10 LP cells. Two-dimensional gel

electrophoresis of newly synthesized cellular and mitochondrial proteins has yet to provide a simple relationship between any particular peptide and steroid biosynthesis. Together these data suggest that hCG stimulation results in changes in the mitochondria of the parent stock which were not induced to the same extent in the mitochondria of MA-10 LP.

LIST OF TABLES

1.	Effects of inhibitors of pregnenolone metabolism on the secreted steroid profile of MA-10 LP and the parent population	30
2.	22R-hydroxycholesterol stimulated side-chain cleavage capacity in parent and MA-10 LP cells and isolated mitochondria	32
3.	Cholesterol side-chain cleavage activity in isolated mitochondria from parent and MA-10 LP	33
4.	SCP ₂ stimulated steroid production by isolated mitochondria	43
5.	Effects of inhibitors of intracellular calcium on hCG stimulated steroid production in whole cells	45
6.	Effects if inhibitors of intracellular calcium on hCG stimulated steroid production in isolated mitochondria	47
7.	Total integrated density of proteins 1 through 7 from parent and MA-10 LP mitochondria	58

LIST OF FIGURES

1.	Progesterone production in MA-10 parent stock and MA-10 LP	23
2.	hCG stimulated progesterone production in MA-10 parent stock and MA-10 LP over time in culture	25
3.	Bt ₂ cAMP stimulated progesterone production in MA-10 parent stock and MA-10 LP over time in culture	26
4.	Pregnenolone production in MA-10 parent stock and MA-10 LP	27
5.	Quantitative analysis of 3 β -Hydroxysteroid dehydrogenase activity in MA-10 parent stock and MA-10 LP	29
6.	Quantitative analysis of cytochrome C oxidase activity in MA-10 parent stock and MA-10 LP	35
7.	Total mitochondrial protein isolated from MA-10 parent stock and MA-10 LP	36
8.	Total cAMP accumulation in MA-10 parent stock and MA-10 LP following hCG stimulation	37
9.	Total cAMP in MA-10 parent stock and MA10 LP following hCG stimulation	38
10.	Total cAMP dependent protein kinase A activity	40
11.	Functional integrity of mitochondria isolated from MA-10 parent stock and MA-10 LP	42
12.	Effects of cycloheximide treatment of whole cells on mitochondria isolated from MA-10 parent stock and MA-10 LP	48

13.	Effects of mitochondrial sonicates from control and stimulated parent and MA-10 LP cells on intact mitochondrial steroid production from unstimulated parent cells	49
14.	Effects of mitochondrial sonicates from control and stimulated parent and MA-10 LP cells on intact mitochondrial steroid production from unstimulated MA-10 LP cells	50
15.	Dose dependent effects of mitochondrial sonicates from MA-10 parent stock and MA-10 LP on intact parent mitochondria	52
16.	The effects of hCG and Bt ₂ cAMP on the synthesis of mitochondrial proteins in MA-10 parent cells	54
17.	The effects of hCG and Bt ₂ cAMP on the synthesis of mitochondrial proteins in MA-10 LP cells	56

CHAPTER I

INTRODUCTION

Historical

The study of the structure and function of the testis has historically been an important aspect of human and animal biology. As early as 15 centuries B.C. the Assyrians castrated men as punishment for sexual offenses (3) implying that the effects of castration on libido and reproductive competence was recognized at least by this time. Knowledge of the effects of castration in animals is even more ancient, perhaps dating back as far as 7000 B.C. to the Neolithic age when animals were domesticated (4). The first clear evidence of an endocrine role of the testis was provided by Berthold in 1849 (5). Berthold demonstrated that loss of the comb in roosters following castration could be prevented if the testis were transplanted to an abnormal site. Since any nervous connection would be severed during transplantation, he concluded some 50 years before the terms hormone and endocrine were proposed that a blood-born factor must be provided by the testis which could affect distant tissues (6). A year later Franz Von Leydig described interstitial cells in the testis of several mammals and Bouin and Ancel in 1903 (7) provided evidence for an endocrine role of these Leydig cells. In 1889 the French physiologist Brown-Séquard, then in his seventies, reported dramatic increases in sexual activity following self-injection of extracts of animal testis (8). These and other studies provided the basis for the ongoing study of reproductive function and reproductive endocrinology.

Endocrine hormones are the blood-born regulators of cellular function which modulate the actions of various organs in a physiologically coordinated manner including reproductive competence. These chemical messengers are most commonly peptide or steroidal in nature and regulate some aspect of the phenotypic expression of virtually every cell in the body. The synthesis and secretion of these hormones is controlled either by direct

neural stimulation which induces the release of peptide hormones from the posterior pituitary or by factors released from the hypothalamus into the portal circulation which stimulates the release of peptide hormones from the anterior pituitary. These pituitary peptide hormones are released into the systemic blood supply to exert their effects directly on target cells. In addition, some hormones from the anterior pituitary stimulate the synthesis and release of steroid hormones which themselves regulate the phenotypic expression of target tissues.

The Testis

The testis is comprised of two physiologically distinct compartments, the interstitial space and the lumen of the seminiferous tubules which are functionally separated by the blood-testis barrier (9, 10). The Leydig cells, which comprise about 12 % of testicular volume in humans (11), are found in the interstitial compartment of the testis along with macrophages, fibroblasts and other components commonly found in loose connective tissue. Encircling the seminiferous tubules are the peritubular myoid cells. Within these tubules are found the Sertoli cells and the continuum of stages from germ cell to spermatozoa. Specialized tight junctions connect adjacent Sertoli cells effectively preventing the passage of substances in between these cells. It is these tight junctions which are believed to be integral in the formation of the blood-testis barrier and requires substances entering the luminal compartment to pass through the Sertoli cells (12-14). In the prepubertal testis of the human (3 to 8 years of age) regions of interdigitation exists between apposing membranes of adjacent Sertoli cells, but the typical junctional complexes composing the blood-testis barrier are not yet present (15). During this stage of development, lanthanum tracer will penetrate through the Sertoli cells and reach the lumen of the seminiferous tubule, indicating that the blood-testis barrier is not yet functional. At the time of puberty (11 to 13 years of age) the tight junctions have formed and the penetration of lanthanum is blocked suggesting a

temporal arrangement of the occurrence of a functional blood-testis barrier and the completion of spermatogenesis. This and other data has lead to the conclusion that it is the Sertoli cells which regulate the unique tubule environment necessary for spermatogenesis (16). While it is now known that other hormones play a role in normal testicular physiology and Sertoli cell function, it is clear that spermatogenesis cannot occur in the absence of the male steroid hormone testosterone (17-23).

Regulatory Mechanisms

In the male the hormones which mediate the process of sexual dimorphism and reproductive functions are the steroid hormones collectively known as androgens. Androgens are responsible for the development and maintenance of the internal genitalia, the appearance of the secondary male sexual characteristics, the development of the male skeletal muscle phenotype, feedback inhibition of the hypothalamus pituitary axis and stimulation of spermatogenesis (24). Additionally, androgens stimulate the skin and the kidneys as well as some aspects of central nervous system function (25). The source of testicular androgens was initially based on studies which provided circumstantial evidence, largely morphological, suggesting that the interstitial cells were the source of these steroids (26, 27). The first direct evidence indicating that the source of androgens came from studies which were able to localize the activity of the steroidogenic enzyme 3β -hydroxysteroid dehydrogenase (3β -HSDH) in Leydig cells (28, 29). The possibility that these androgens might be synthesized by the seminiferous tubules was eliminated by Christensen and Mason who demonstrated that isolated interstitial cells were the main source of these androgens (30). Although the principle androgen synthesized by the male is testosterone other steroids which display varying degrees of androgenic activity include estradiol, dihydrotestosterone, androstenedione and dehydroepiandrosterone (31).

The neuro-endocrine regulation of testosterone synthesis is controlled at the level of the pituitary by the pulsatile secretion of gonadotrophin releasing hormone (GnRH) into the portal blood supply from GnRH neurons in the medial preoptic area. GnRH binds to surface receptors on cells known as gonadotrophs located in the anterior pituitary which results in the increased synthesis and pulsatile secretion of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH). LH and FSH are released from the gonadotrophs in a process which is mediated by a rise in intracellular free calcium (32-35). LH is released into the blood and eventually binds to specific cell surface receptors of the Leydig cells (36) resulting in the synthesis and secretion of testosterone (37). Testosterone functions in the phenotypic expression of the secondary male sexual characteristics mentioned above as well as in the maintenance and support of spermatogenesis. Testosterone can also feed back to the level of the hypothalamus and pituitary to inhibit the secretion of LH from the pituitary gonadotrophs (38).

Biochemical Mechanisms of LH Action

Steroid hormones are synthesized from cholesterol and it is the mobilization of this precursor which has been the focus of numerous studies designed to understand the regulation of steroid biosynthesis. The cholesterol which is used for steroid hormone production can be synthesized de novo from acetate or taken up by cells from plasma sources (39, 40) and stored as free cholesterol or esterified (41) in lipid droplets or plasma membranes (42). Following stimulation, cholesterol is mobilized from cellular stores and transported to the mitochondria in a process mediated by microfilaments. This has been determined using inhibitors of actin such as cytochalasin B (43) which prevents the hCG stimulated transport of cholesterol to the mitochondria (44-49). Additional evidence that actin functions in the transport of cholesterol in steroidogenic tissues comes from liposomal fusion experiments (50) which demonstrated that

anti-actin antibodies (51-53) also blocked cholesterol transport to mitochondria and decreased the synthesis of steroid in these cells (54). Other proteins such as sterol carrier protein 2 (SCP₂) may also be involved in this process (55-62). The delivery of cholesterol from cellular stores to the outer mitochondrial membrane is known to take place independent of new protein synthesis as inhibitors of translation such as cycloheximide will not inhibit this transport (63). Following the delivery of cholesterol to the outer mitochondrial membrane, cholesterol is transported from outer to inner mitochondrial membrane in a cycloheximide sensitive process (63) where it becomes available as a substrate for the cholesterol side-chain cleavage complex (CSCC). This complex consists of three enzymes, adrenodoxin, adrenodoxin reductase and cytochrome P450_{SCC}, and catalyzes the conversion of cholesterol to pregnenolone and isohexanoic acid (64-66). Although the conversion of cholesterol to pregnenolone is the rate-limiting enzymatic step, an increasing body of evidence suggests that it is the delivery of cholesterol from outer to inner mitochondrial membrane which is the true rate-limiting step in steroid biosynthesis (1, 67-68).

From model experiments using purified cholesterol side-chain cleavage enzyme complex reconstituted in lipid membranes, the interaction between cholesterol and the cytochrome P450_{SCC} appeared to be under the influence of the phospholipid composition of the membrane bilayer (69-78). Trophic stimulation of adrenal and thecal cells resulted in the alteration of mitochondrial membrane phospholipid composition which was demonstrated as a rise in phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine (79-80) and these changes were blocked by pretreating cells with cycloheximide (81). To elucidate the role of these changes in isolated mitochondria, purified mitochondria were collected from stimulated cells, phospholipids extracted and purified and these fractions were placed back on mitochondria isolated from unstimulated cells. Addition of mitochondrial phospholipids prepared from stimulated cells resulted in a significant increase in the steroid output of mitochondria

isolated from unstimulated cells, whereas phospholipids from unstimulated mitochondria were without effect (82-83). These results suggested that the alterations in mitochondrial phospholipid composition which occur following trophic stimulation can affect steroid output and may be mediated by a newly synthesized protein.

In adrenal and ovarian tissue ongoing protein synthesis is required for tropic induction of steroid hormone synthesis in Leydig cells (84). This has lead to an extensive and as yet inconclusive search for an induced protein or proteins which can stimulate the production of steroid by intact cells or isolated mitochondria. From studies using the protein synthesis inhibitor cycloheximide this protein is suspected to have a half life of 11.5 min following acute stimulation (85). While still not unequivocally determined, several candidates for this obligatory protein(s) have been described (86-90). The functional role this protein(s) must perform remains obscure although direct activation of the cholesterol side-chain cleavage enzymes, as reported with Pedersen and Brownie's steroidogenic activator peptide (SAP), and mediation of cholesterol transport, as is the case with Yanagibashi's 8.2 K protein, are thought to be the most likely points of regulation by this acutely synthesized protein.

Studies on the trophic activation of steroidogenesis in Leydig cells demonstrated that cAMP fulfills three criteria of a second messenger. hCG binds to specific cell surface receptors which stimulates the synthesis of cAMP (91), cAMP analoges such as Bt₂cAMP stimulate steroid biosynthesis and inhibition of phosphodiesterase, which inactivates cAMP, potentiates the stimulatory effects of low concentrations of hCG on steroid production (92). Additionally, the diastereoisomers of adenosine 3',5'-cyclic phosphorothioate, (Sp)-cAMP and (Rp)-cAMP which have been shown to act as agonists and antagonists, respectively, of cAMP-dependent protein kinases (PK-A) in several mammalian species have been used to explore the role of cAMP in the stimulation of steroid production. Pereira et al. (93) have shown in cultured MA-10 mouse Leydig tumor cells that (Sp)-cAMP

stimulates steroid production by Leydig cells while (Rp)-cAMP inhibits hCG stimulated steroid synthesis. Furthermore, removal of the surface bound hormone resulted in the cessation of cAMP production and steroid hormone synthesis (94). Together these data indicate that while other signal transducing pathways may be stimulated in Leydig cells following LH/CG stimulation a rise in intracellular cAMP levels is sufficient to stimulate testosterone synthesis by Leydig cells (95-96). Additionally, Pereira et al. concluded that if other second messengers are involved in the activation of steroidogenesis by LH/CG, they must do so by acting together with, rather than independently of, cAMP (93).

The role of calcium in the regulation of steroid production has recently been investigated following the observation that LH/CG stimulation of Leydig cells results in an increase in free cytosolic calcium (97-98). Interestingly, Davis et al. (99) observed no increase in free cytosolic calcium levels in Bt₂cAMP stimulated luteal cells suggesting that the mobilization of calcium in LH/CG stimulated Leydig cells is activated directly by receptor mediated events and not as a consequence of the accumulation of cAMP. It has also been observed that low levels of LH can be used to stimulate a measurable rise in testosterone secretion and free cytosolic calcium in Leydig cells without eliciting a detectable rise in cAMP (100). Removal of extracellular calcium or addition of calcium channel blockers have been shown to reduce the steroidogenic response in Leydig cells (101-103) while liposomes containing calcium enhanced steroid synthesis, increased the transport of cholesterol to the mitochondria and increased the activity of the cholesterol side-chain cleavage enzyme (104). Taken together these results raised the possibility that the control of gonadal steroidogenesis by LH/CG may involve the activation of cAMP dependent phosphorylating pathways as well as calcium signalling pathways (105).

Investigation of the cellular events involved in the regulation of steroid production by Leydig cells has lagged behind studies performed in thecal and adrenal cells due to technical difficulties associated with cell

isolation procedures. To aid in the study of trophic regulation of steroid hormone biosynthesis, cloned cell lines have been isolated which can be grown in large quantities and are free from contaminating cells which plague primary cell cultures and can make interpretations of experimental results difficult. We have chosen to use a mouse Leydig tumor cell line, MA-10 cells, which responds to LH and hCG stimulation by synthesizing progesterone as its major steroidogenic endproduct (106). This cell line was cloned from the solid M5480P tumor and adapted to growth in culture by alternately growing them in culture and host animals (107). Since their development, MA-10 cells have been used to study the action of gonadotrophins (108-109), receptor dynamics (110-111), cholesterol compartmentalization and mobilization (42, 112), the action of nongonadotrophic hormones on steroid production (95, 113) and trophic induction of protein synthesis (89).

In the present study we report the isolation and characterization of a subclone of the MA-10 (MA-10 LP) cell line (1-2). Cell lines have been reported to lose their ability to synthesize steroid over time in culture in response to hormone stimulation (114). We have observed a similar decrease in the responsiveness of MA-10 cells with time in culture. This observation coupled with other indications that MA-10 cells may be a heterogeneous population prompted an attempt to isolate subclones of this cell line. From the initial 6 subclones isolated 1 clone was chosen for further study based on the observation that it produced low levels of progesterone in response to maximal hCG stimulation. We reasoned that elucidation of the lesion which attenuates the steroid response could possibly lend insight into the regulation of steroid hormone biosynthesis. Therefore, the aim of these studies was to determine the site of the lesion(s) in MA-10 LP which distinguishes it from the MA-10 parent stock and results in an diminished steroidogenic response. The results presented in this work demonstrate that the lesion in MA-10 LP lies distal to the production of cAMP, proximal to cholesterol side-chain cleavage activity and is not due

to a defective sterol carrier protein 2. While the activation of steroid biosynthesis requires a rise in intracellular free calcium, the intracellular stores of calcium are sufficient for this activation and does not appear to be responsible for the lesion in the low progesterone secreting subclone. The activation of cellular steroid synthesis by both cell types is retained in isolated mitochondria when incubated in a cell-free system and the activation of these mitochondria required ongoing protein synthesis, is mediated by the calcium-calmodulin complex and was greater in the parent than in MA-10 LP. Sonicates from hCG stimulated parent cell mitochondria were able to stimulate steroid production in control mitochondria whereas similarly treated clone LP mitochondria sonicates were not. This indicated that the lesion in clone LP resulted from an inability of trophic hormone treatment to make the necessary alterations in the mitochondria which were required for subsequent steroid production. Mitochondrial protein profiles of hormone treated cells demonstrated that significant differences in the patterns of newly synthesized proteins exists between these two clones.

CHAPTER II

MATERIALS AND METHODS

Chemicals

Waymouth's MB 752/1 medium, horse serum, Dulbecco's phosphate-buffered saline without calcium or magnesium (PBS⁻), lyophilized trypsin-EDTA and tissue grade sodium bicarbonate were purchased from GIBCO (Grand Island, N.Y.), glass fiber filters were obtained from Watman (Clifton, NJ) and cryotubes were procured from NUNC (Neptune, NJ). Dulbecco's phosphate buffered saline containing calcium and magnesium (PBS⁺) was obtained from Oxoid Ltd. (Basingstoke, England) and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from Research Organics, Inc. (Cleveland, Ohio). Cycloheximide, N⁶-2'-O-dibutyryladenine-3':5'-cyclic monophosphate (Bt₂cAMP), 3':5'-cyclic monophosphate (cAMP), Coomassie blue G-250, ethylenediamine-tetraacetic acid (EDTA), gentamycin sulfate, bovine serum albumin (BSA), pimozone, 3,4,5-trimethoxy benzoic acid 8-(diethylamino) octyl ester (TMB-8), pregnenolone, testosterone, progesterone, 3-isobutyl-1-methylxanthine (IBMX), Hoechst 33258 stain (benzoxanthine), β -nicotinamide adenine dinucleotide (NAD⁺), dimethyl sulfoxide, 22R-hydroxycholesterol (22R), DL-isocitrate, cytochrome c, ascorbate, CHAPS (3-[(3-cholamidopropyl) dimethylammonio] 1-propanesulfonate), trichloroacetic acid (TCA), sodium phosphate, DL-dithiothreitol (DTT) and the reduced form of β -nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Co. (St. Louis, MO). SCP₂ was provided by Dr. K.W.A. Wirtz and was prepared according to the method of Teerlink et al. (1984) while dextran T-70 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Carbon decolorizing (Norite) was purchased from Fisher Scientific (Fair Lawn, N.J.), cyanoketone from Sterling-Winthrop (Rensselaer, N.Y.) and SU-10603 was obtained from CIBA-GEIGY (Summit, N.J.). hCG was obtained

from the National Hormone and Pituitary Program, NICHD, NIH, while [7-³H(N)] pregnenolone [1 α , 2 α -N-³H]testosterone, [26-¹⁴C]cholesterol and [1,2,6,7-N³H] progesterone were purchased from New England Nuclear (Boston, MA). [³⁵S]methionine-cysteine (Trans Label), Aquasol and [2,8-³H]-cAMP was purchased from ICN Radiochemicals (Irvine, CA). Ampholines were obtained from Pharmacia-LKB Biotech Inc. (Piscataway, NJ) while other chemicals used in the preparation of isoelectric focusing gels were obtained from BioRad (Richmond, CA). 10-20% gradient gels were purchased pre-cast from Integrated Separation Systems (Hyde Park, MA) while glass fiber and cellulose phosphate filters were obtained from Millipore (Bedford, MA). Progesterone and testosterone antibody were purchased from Holly Hill Biological (Hillsboro, OR) and pregnenolone antibody was provided by Dr. F.F.G. Rommerts (Erasmus University, Rotterdam, The Netherlands).

Cell Culture

The MA-10 cells used in these experiments were generously provided by Dr. Mario Ascoli of The Population Council, Rockefeller University and were maintained and subcultured using published techniques (106). Cells were routinely grown in 75 mm² T-flasks in Waymouth's medium supplemented with sodium bicarbonate, HEPES buffer, gentamycin sulfate and 15% heat inactivated horse serum. For most experiments cells were plated into 6, 12 or 24 multiwell tissue culture dishes or in 100 mm² culture dishes (Corning) and grown for 24 h in medium containing 15% horse serum. At the beginning of an experiment cells were washed twice with PBS⁺ and medium lacking serum but containing appropriate treatments was placed back on the cells. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Trypsin dispersed cells were counted using a model ZF Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida) and protein was measured by the Coomassie blue G-250 binding method (115) using BSA as a standard. As cell lines have been shown to loose

responsiveness over time in culture, cells were routinely thawed from frozen stock every 2 to 3 months. When freezing cells, trypsin-dispersed cells were suspended in ice-cold Waymouth's medium containing 15% horse serum and 10% glycerol, transferred to cryotubes and placed on ice for 30 min. Tubes were transferred to a Styrofoam cup, packed with paper towels, inverted several times to resuspend cells and placed in a -80°C freezer overnight. Cells were then transferred to liquid nitrogen tanks for long term storage. To thaw cells cryotubes were submerged in a 37°C bath and agitated until completely thawed. Vials were washed thoroughly with 70% ethanol under a sterile hood, dried and the contents poured into a T-75 flask to which warm medium containing 15% horse serum had been added. Following several days of growth, cells were plated into 24-well culture dishes and their steroidogenic response to Bt₂cAMP and hCG tested prior to use.

Cell Cloning

Trypsin-dispersed cells were thoroughly suspended, counted and diluted to a concentration of 0.2 cells/ml medium containing 15 % horse serum to which an equal volume of sterile filtered medium which had been collected from cell cultures (conditioned medium) had been added. To each well of four 24-well tissue culture plates 1 ml of suspended cells was added. After cells had attached, each well was examined and those wells containing only one cell were marked and reexamined every 24 h. After 2 to 3 weeks of growth these clones had grown to sufficient numbers to allow testing of their steroidogenic responses. Six subclones were identified which exhibited various steroidogenic responses to hCG and Bt₂cAMP stimulation. Each of these cell populations were then recloned as described above to ensure that monoclonal populations had been obtained. Following the second cloning, hCG stimulated steroid production in all isolated cell populations was re-

examined and compared to that secreted by the parent stock. Of these six cell populations two were chosen for further characterization based on their steroidogenic profiles. Cells were routinely monitored for mycoplasma contamination according to the method of Hessling et al. (116). Following staining, cytoplasmic DNA was visualized using the fluorescent Hoechst 33258 stain and compared with both positive and negative controls.

RIA

RIA analysis of testosterone, pregnenolone and progesterone was performed essentially as described previously (1, 117) on aliquots of medium collected from control and treated cells or on aliquots of cholesterol side-chain cleavage (CSCC) buffer (125 mM sucrose, 25 mM tris-HCl, 5 mM MgCl₂, 60 mM KCl, pH 7.4) after pelleting mitochondria. Inhibitors of pregnenolone metabolism, cyanoketone and SU-10603 were added, where indicated, at 5 and 20 μ M, respectively. For standard curves steroids were dissolved in ethanol, appropriate volumes pipetted into 12 by 100 mm tubes and blown to dryness under air. Samples were added to duplicate tubes and medium or CSCC buffer added to standard, non-specific binding and recovery tubes and shaken in a 37°C water bath. Tracer and antibody were added and incubated for 15 min then placed on ice. To stabilize the antibody-steroid complex 100 μ l phosphate buffered saline containing 0.5% gelatin (PBS-G) was added. Following a 15 min incubation on ice, dextran-coated charcoal was added to adsorb any unbound steroid and pelleted at 600 x g for 10 min. The supernatant was then poured into scintillation vials, aqueous cocktail added and assayed for radioactivity. Analysis of data was made using the RIA analysis program provided by Dr. Reid Norman of this center on an Apple III computer and expressed as the amount of steroid secreted per 10⁶ cells or per mg protein.

cAMP Binding Assay

cAMP levels were measured as described by Gilman (118) as modified by Tovey et al. (119). Cells were plated into 12 well plates at 10^6 cells per well, grown 24 h, washed twice and medium lacking serum but containing appropriate treatments was placed back on cells. Each well was washed twice with PBS⁻ and cAMP extracted with 5% TCA. Extracts were vortexed, precipitated proteins pelleted and the TCA was removed with two water saturated ether washes. The aqueous phase was then placed into cryo tubes, frozen to -80°C and lyophilized overnight. Samples were dissolved in cAMP buffer (10 mM Tris-HCl, 4 mM EDTA, pH 7.4) and cAMP quantitated using a binding protein assay and data analyzed on the same computer program as that described for RIA analysis. Mouse calvaria protein kinase was prepared from 4-day-old neonatal mouse heart by homogenization in cAMP assay buffer and centrifuging samples at $17,000 \times g$ for 10 min. The supernatant containing the cAMP binding protein was then stored in 5 ml aliquots until use. Dilutions of the binding protein were tested on standard cAMP curves to determine the dilution factor required to yield 50% binding. The binding protein was kindly provided by Dr. John A. Yee of the Department of Cell Biology and Anatomy of this center. The phosphodiesterase inhibitor IBMX was employed in cAMP accumulation studies at a final concentration of 0.4 mM.

Quantitative 3β -Hydroxysteroid Dehydrogenase Assay

Quantitation of 3β -HSDH activity was performed by a modification of the method described by Wiebe (120). 3β -HSDH catalyzes the reaction which converts pregnenolone to progesterone. Homogenates were prepared from cultures which were grown 24 h in medium containing horse serum at which time cells were washed twice with PBS⁻, scraped from the plastic culture dishes and homogenized in Tris-sucrose-EDTA. Whole cell

homogenates were centrifuged at $600 \times g$ and cytosolic preparations were kept on ice until use. 3β -HSDH buffer (0.1 M sodium phosphate, pH 8.0) was prepared containing 27 mM NAD^+ and 172 μM isoandrosterone which serves as substrate and the source of hydrogen ions in the reduction of NAD^+ . Cytosolic preparations were added and the reduction of NAD^+ to NADH ($\epsilon=6.22/\text{mmol}$) was measured spectrophotometrically at 340 nm over ten minutes at room temperature. Substrate dependent cytosolic 3β -HSDH activity was determined by measuring the endogenous NAD^+ reductase activity and subtracting this from the reductase activity in the presence of isoandrosterone (86). Data are expressed as the mean micromoles of NAD^+ reduced per min per mg cytosolic protein from three separately collected cytosolic homogenates ($\pm\text{SD}$).

Cytochrome C Oxidase Assay

Cytochrome c oxidase activity (E.C. 1.9.3.1.), a marker protein for inner mitochondrial membranes, was measured on crude cellular homogenates and on isolated mitochondria (121-122). Twenty-four-h cultures were washed twice with PBS⁻, trypsin dispersed and aliquots removed for cell counts. The remaining cells were then placed into 13 by 100 mm tubes containing Tris-sucrose-EDTA, centrifuged at $600 \times g$ for 10 min and the supernatant discarded. The whole cells were suspended in 10 mM sodium phosphate buffer and homogenized in a Potter-Elvehjem glass homogenizer with a teflon pestle for 25 strokes at 1000 RPM. Mitochondria were isolated from 24-h-old cultures as described and suspended in 10 mM sodium phosphate buffer, pH 7.1 and aliquots removed for protein and cytochrome C oxidase activity. Aliquots of crude cellular homogenates and isolated mitochondria were added to a solution of 50 μM cytochrome c and 50 μM ascorbate. The oxidation of cytochrome C ($\epsilon=29.5/\text{mmol}$) was measured as the decrease in absorbance at 550 nm over a 10-min period at room temperature. In cellular homogenates data were expressed as μmols

cytochrome c oxidized per min per 10^6 cells from three separate cell cultures $\pm$ SD while in isolated mitochondria data were expressed as μ moles cytochrome c oxidized per min per mg isolated mitochondrial protein from three separate cell cultures $\pm$ SD.

Cholesterol Side-Chain Cleavage Assay

CSCC activity was measured using a method described by Chaudhary and Stocco (123). Briefly, isolated mitochondria (89) were incubated in the presence of [26- 14 C]cholesterol and NADPH as reducing equivalent at 37°C for 2 h. [14 C]Isohexanoic acid, one product of the cholesterol side-chain cleavage reaction, was separated from uncleaved cholesterol following acidification of the aldehyde forms by chloroform extraction and the labeled product in the aqueous phase was counted on a Beckman 7800 LSC (Beckman, Palo Alto, CA) using Aquasol as scintillation cocktail. This procedure was demonstrated to recover 99% of the isohexanoic acid, while virtually none of the cholesterol remained in the aqueous phase (123).

A second method of measuring CSCC capacity not limited by intracellular cholesterol was accomplished by incubating cells and isolated mitochondria in the presence of the hydroxylated CSCC intermediate 22R hydroxycholesterol (22R). This is a natural intermediate in the CSCC reaction and its movement in the mitochondria does not appear to be regulated as is the case with endogenous cholesterol. Incubation of MA-10 cells in the presence of 50 μ M 22R results in the accumulation of progesterone (1) and similar results have been published for 22R-stimulated primary mouse Leydig cell cultures (124). Isolated mitochondria were incubated in CSCC buffer containing 22R (50 μ M), SU-10603 (20 μ M) and cyanoketone (5 μ M) at 37°C for 3 h with or without isocitrate which serves as a substrate in the generation of reducing equivalent. Reactions were terminated by immersing tubes in a dry ice-methanol bath. After thawing,

mitochondria were pelleted and aliquots of the supernatant removed for RIA analysis of progesterone. Progesterone was also extracted from the CSCC buffer into chloroform (125), blown to dryness under nitrogen and redissolved in Waymouth's medium for RIA analysis. Evaluation of these methods demonstrated that the extractable progesterone did not differ from that measured directly on aliquots of CSCC buffer (data not shown); therefore, all subsequent analysis was performed directly on the CSCC buffer.

Protein Kinase A Activity

cAMP dependent protein kinase (PK-A) activity was measured essentially as described by Gill and Walton (126) on cell homogenates. Briefly, cells were grown 24 h in medium containing horse serum, washed twice with Tris-sucrose-EDTA, scraped from the plastic and homogenized at 1000 rpm with 15 strokes with a Potter-Elvehjem glass-teflon tissue grinder. After removing samples of cellular homogenates for determination of protein concentration aliquots were then incubated in the presence or absence of cAMP in kinase buffer (300 mM KH_2PO_4 , 20 mM DTT, pH 6.8) containing $[\gamma\text{-}^{32}\text{P}]$ ATP as a phosphate donor and histone H2b as a nonspecific phosphate acceptor. Reactions were carried out at 37°C for 30 min, then terminated by adding 20% TCA and protein precipitated on ice for 1 h. Precipitated proteins were then collected on cellulose phosphate paper (P81), dried under a heat lamp, placed into scintillation vials, non-aqueous scintillation cocktail added and the radioactivity assessed. The total CPM in the precipitated protein in the presence of cAMP minus that in the absence of exogenous cAMP was used as a measurement of the total PK-A activity. Data were expressed as the CPM per μg protein.

Isolation of Mitochondria

Intact mitochondria were isolated from 24-h-old control and hormone stimulated cells. Cultures were washed twice with medium lacking horse serum and medium lacking serum but containing appropriate inhibitors was

placed back on cells 10 min prior to hCG (34 ng/ml) addition. Following a 1-h incubation in the presence or absence of hCG, cells were washed twice with PBS⁻, scraped from the dishes and pelleted at 600 × g for 10 min. Cells were resuspended in 5 ml of isolation buffer (0.1M Tris-HCl, 0.25 M mannitol, 1 mM EDTA, pH 7.4) and homogenized by hand for 10 strokes (5 sec on the up strokes and 5 sec on the down strokes) using a glass-glass Wheaton 7 ml dounce tissue grinder with a 0.001-0.003" clearance. Homogenates were centrifuged at 600 × g, the mitochondria pelleted from the initial supernatant by centrifugation at 17,000 × g for 10 min and resuspended in CSCC buffer. Where indicated, SCP₂ was added to yield a 1 μM final concentration. Aliquots (100 μl) were then transferred to 1.5 ml microfuge tubes (Denville Scientific, Denville, N.J.) containing 400 μl incubation buffer (CSCC buffer containing a final concentration of 10 mM isocitrate) and incubated for 30 min in a 37°C shaking water bath. Reactions were terminated by submersing the microfuge tubes in a dry ice-methanol bath. After thawing, samples were centrifuged at maximum speed in a Beckman Microfuge 12 for 15 min to pellet the mitochondria and samples stored at -20°C until steroid was quantitated by RIA directly on the supernatant fraction. To inhibit pregnenolone metabolism SU-10603 (20 μM) and cyanoketone (5μM) were added where indicated to inhibit 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase, respectively (127).

Mitochondrial sonicates were prepared from cells grown as described above and homogenized in isolation buffer using a Thomas glass-teflon homogenizer at 1000 RPM for 15 strokes. Mitochondria were then isolated by differential centrifugation as described, resuspended in CSCC buffer and sonicated in an ice bath using the microtip on a Tekmar sonic disruptor at 10% duty cycle, with an output control of 5, for 15 pulses on the pulse setting. Sonicates were kept on ice while intact mitochondria were isolated. Following isolation of the mitochondria by differential centrifugation as described, incubations were set up containing intact mitochondria, 10 mM isocitrate, 20 μM SU-10603, 5 μM cyanoketone and varying amounts of

sonicated mitochondrial proteins. Following a 30-min incubation, the mitochondria were pelleted as described and the supernatants assayed for pregnenolone by RIA.

Radiolabelling of Cells

Radiolabelling of mitochondrial proteins was accomplished by growing 2 million cells in a 6-well culture dish for 24 h. Cells were washed twice then 500 μ l of Waymouth's medium containing 1.0 mCi/ml 35 S-methionine-cysteine and the appropriate hormone treatment was placed back on cells. At the end of the 6-h labelling period, the medium was removed, the cells washed three times with PBS⁻ and scraped with a rubber policeman in the presence of homogenization buffer (0.1 M Tris-HCl, 0.25 M sucrose, 1 mM EDTA, pH 7.4). Cells were transferred to a Potter-Elvehjem motor-driven glass homogenizer with a teflon pestle. Each sample was homogenized at 1000 RPM for 25 strokes. Mitochondria were isolated by differential centrifugation as described (89) and 200 μ l of isoelectric focusing buffer (IEF lysis buffer; 9.8 M urea, 2% CHAPS, 2% ampholines and 100 mM dithiothreitol) was carefully layered over the pellets and remained at room temperature for 24 to 72 h prior to vortexing. To determine the amount of radioactivity in each sample, 10 μ l aliquots of mitochondrial lysates were precipitated with 20% trichloroacetic acid (TCA) at 4°C for 2 h. Precipitates were collected on glass fiber filters (Millipore), washed extensively with 5% TCA, dried under a heat lamp, filters placed into scintillation vials, cocktail added and assayed for radioactivity in a Beckman liquid scintillation counter.

Two-Dimensional Gel Electrophoresis

Two dimensional gel electrophoresis was performed on mitochondrial proteins essentially as described (89, 128-129). Isoelectric focusing (IEF) tube gels were poured into 1.5 mm by 13 cm tubes to a height of 10.5 cm, 200 μ l of overlay buffer (8 M urea, 1% 3.5-9.5 ampholines) added and allowed to polymerize for 2 to 4 h. Tube gels were then pre-focused at

200 volts for 15 min, 400 volts for 30 min and 500 volts for an additional 30 min prior to removing overlay buffer and adding equal amounts of TCA-precipitable CPM from each sample. Gels were focused at 500 volts for 17 h and 1000 volts for an additional h. pH gradients of the IEF gels were determined on a IEF gel which received no sample using a surface electrode (Orion Research, Boston, MA). Gels were removed, placed in equilibration buffer (10% glycerol, 2.3% SDS and 6.25 mM tris, pH 6.8) for 8 min and placed on top of precast 10 to 20% gradient slab gels. Radioactive molecular weight protein standards (NEN, Boston, MA) were included in each set of gels. Gels were run at 37.5 mAmps per gel, placed in fix overnight (50% ethanol, 5% glacial acetic acid and 45% water) then washed with four changes (once every hour) of degassed, distilled water. Gels were prepared for fluorography using Resolution (E.M. Corp., Chestnut Hill, MA), dried under moderate vacuum for 3 h (30 min without heat, 2 h at 55°C then an additional 30 min without heat) and exposed to X-ray film at -80°C for an appropriate length of time in light tight Kodak X-Omatic Cassettes (Eastman Kodak Co., Rochester, NY).

Quantitation of Proteins on Gels

Proteins of interest were quantitated using a BioImage Visage 2000 (BioImage, Ann Arbor, MI) computer assisted analysis system. Briefly, X-ray images of 2-D PAGE protein profiles were scanned, captured and adjusted for differences in the amount of radioactivity loaded onto gels by quantitating a number of proteins (12 to 25 proteins per gel) which do not change following stimulation. The ratio of the integrated optical densities of these proteins were computed and used in normalizing gels for comparison. In this manner proteins of interest could be compared after normalizing all spots for differences which can be generated on the basis of loading. The proteins described in this study were quantitated using a comparative log software program and the results reported as the total integrated optical density of each spot following normalization of gels.

Statistical Analysis

All statistical analysis were performed on an IBM PC with a program written by Dr. Charles Garner of this center. The test utilized in these studies was the Student's t-test for comparison between means. In the cases where more than two means were compared the Newman-Keuls test was applied. This program was also written by Dr. Charles Garner. Where relevant, levels of statistical significance are indicated in the text and figure legends.

CHAPTER III

RESULTS

Mycoplasma

Following the initial cloning of these cells, again after the secondary cloning and following the initial thawing of frozen cultures each clone was tested for mycoplasma infection. During the course of the studies outlined in this dissertation never did any of these subclones test positive for cytoplasmic DNA staining which is indicative of mycoplasma infections.

Production of Steroid

Following the initial subcloning of the MA-10 cell line, six populations were identified which secreted varying amounts of steroid following hCG and cAMP stimulation. These cell lines were then cloned again to ensure that monoclonal populations were obtained. Following stimulation by maximal doses of hCG or Bt₂cAMP, RIA analysis of the steroid secreted into the medium by isolated subpopulations indicated that two of the clones produced very different amounts of progesterone, the predominant steroidogenic end product of MA-10 cells (Fig. 1). Based on the levels of progesterone secreted, one of the clones (MA-10 LP) was selected for further characterization and the cellular mechanisms of steroid hormone synthesis compared to that of the other cloned lines. While the original parent population secreted less steroid compared to the highest progesterone producing subclone, we continued to use this monoclonal population and have referred to this as the parent population throughout these studies. In characterizing the lesion which resulted in the diminished steroid secreting ability of MA-10 LP to respond to hormone stimulation we hoped to gain insight into the acute regulation of steroid hormone biosynthesis.

Figure 1. Progesterone production in MA-10 parent stock and MA-10 LP

Following 24 h in culture cells were washed twice, medium lacking horse serum but containing appropriate treatments was placed back on cells and incubated for 1 h at 37°C. Medium was collected for RIA analysis of progesterone while trypsin dispersed cells were counted. All points represent the mean of four separate determinations of individually treated cell cultures $\pm$ SD. While hCG (34 ng/ml) or Bt₂cAMP (1mM) stimulation significantly increased progesterone production by both cell populations, parent cells secreted significantly more steroid than did MA-10 LP ($P < 0.001$).

To determine if the lower steroid output in MA-10 LP could be a result of the time required to respond to stimulation, progesterone accumulation was measured every hour for six h following hCG and Bt₂cAMP induction. As demonstrated in Figures 2 and 3 acute stimulation of MA-10 LP and the parent population resulted in the accumulation of steroid which is maximal by 3 h when stimulated by hCG or Bt₂cAMP. Additionally, the basal rate of secretion remained low throughout the incubation period and did not significantly affect the total accumulation of progesterone by either cell population. Not apparent in these figures, due to the scale necessary to demonstrate the relatively large response seen in the parent, is the modest but significant increase in progesterone production by MA-10 LP following stimulation. In these experiments cells were plated at comparable densities 24 h prior to assessment of steroidogenic capacity. The concentration of cells routinely used resulted in an 80-90% confluent culture. To determine if there were differences in these clones ability to synthesize steroid following stimulation which are density dependent, cells were plated at densities which, following 24 h, resulted in cultures which ranged from 10-100% confluency. The results of these experiments could delineate no differences in the steroid output per million cells by either clone over a wide range of cell densities (data not shown).

The side-chain cleavage of cholesterol results in the formation of pregnenolone while progesterone accumulates as a result of the dehydroxylation of pregnenolone, a reaction which is catalyzed by the enzyme 3 β -hydroxysteroid dehydrogenase (3 β -HSDH). To determine whether the low levels of progesterone synthesis seen in MA-10 LP were due to the low activity of 3 β -HSDH, we examined the amount of pregnenolone secreted by these subclones when stimulated. Figure 4 illustrates that the differences in steroid production by the two cell populations does not appear to be due to the LP clone's inability to convert pregnenolone into progesterone, since there is no significant accumulation

Figure 2. hCG stimulated progesterone production in MA-10 parent stock and MA-10 LP over time in culture

Following 24 h in culture cells were washed twice, medium lacking horse serum but containing hCG (34 ng/ml) was placed back on cells and incubated at 37°C. At 1-h intervals medium was collected for RIA analysis of progesterone while trypsin dispersed cells were counted. All points represent the mean of four separate determinations of individually treated cells cultures $\pm$ SD. Not apparent due to the scale used which is necessary to demonstrate the large response by the parent is the modest but significant stimulation of progesterone production by MA-10 LP ($P < 0.001$).

Figure 3. Bt₂cAMP stimulated progesterone production in MA-10 parent stock and MA-10 LP over time in culture

Following 24 h in culture cells were washed twice, medium lacking horse serum but containing Bt₂cAMP (1mM) was placed back on cells and incubated at 37°C. At 1-h intervals, medium was collected for RIA analysis of progesterone while trypsin dispersed cells were counted. All points represent the mean of four separate determinations of individually treated cells cultures $\pm$ SD. Not apparent due to the scale used which is necessary to demonstrate the large response by the parent is the modest but significant stimulation of progesterone production by MA-10 LP ($P < 0.001$).

Figure 4. Pregnenolone production in MA-10 parent stock and MA-10 LP

Following 24 h in culture cells were washed twice and medium lacking horse serum but containing hCG (34 ng/ml) or Bt₂cAMP (1 mM) was placed back on cells. Cultures were incubated for 1 h at 37°C, the medium was collected for RIA analysis of pregnenolone and trypsin dispersed cells were counted. All points represent the mean from of separate determinations of individually treated cells cultures $\pm$ SD. The scale of this graph is the same as that of Fig. 1 to illustrate the relative amount of pregnenolone which accumulates in stimulated cultures compared to the amount of progesterone which accumulates.

of pregnenolone by either population. It should be noted that the scales in Figures 1 and 4 are the same to aid in the comparison of the total steroid output.

To compare the total activity of this enzyme in cellular homogenates quantitative analysis of the 3β -HSDH activity was performed on each clone. This independent assessment of 3β -HSDH activity demonstrated that there is no difference between the parent stock and MA-10 LP (Fig. 5). Lastly, the low amount of steroid which accumulated following acute stimulation of MA-10 LP is not due to further metabolism of pregnenolone and progesterone to testosterone, as RIA analysis indicated that no measurable testosterone was secreted by either cell population under these conditions (data not shown).

To determine if the accumulation of other steroidogenic intermediates might account for the differences in steroid production between the clones, we employed inhibitors of pregnenolone metabolism SU-10603 and cyanoketone which inhibit 17α -hydroxylase and 3β -HSDH, respectively (127). As these are the only enzymes demonstrated to utilize pregnenolone as a substrate (130), the amount of pregnenolone cleaved from cholesterol by the CSCC complex in the presence of these inhibitors provided a direct measurement of the side-chain cleavage activity. Although progesterone has been reported to be the predominate steroid secreted by MA-10 cells, these cells have been shown to secrete other steroid intermediates which may alter the interpretations of the total steroid secreted by these two cell populations (106). We have, therefore, included both inhibitors in these studies to ensure that we were measuring CSCC activity. Table 1 indicates that there were no differences ($P>0.1$) in the pregnenolone secreted in the presence of these inhibitors as compared to the sum of the pregnenolone and progesterone secreted in the absence of inhibitors. Again this indicated that there was no significant accumulation of pregnenolone by either clone. Additionally, there does not appear to be any significant metabolism of progesterone to other steroidogenic

Figure 5. Quantitative analysis of 3 β -hydroxysteroid dehydrogenase activity in MA-10 parent stock and MA-10 LP

Spectrophotometric quantitation of 3 β -HSDH activity was measured in cellular homogenates using a modification of that reported by Wiebe (120). Twice washed cells were homogenized in Tris-sucrose-EDTA and the conversion of NAD⁺ to NADH measured for 10 min at 340 nm in the presence or absence of 172 μM isoandrosterone as substrate. Data presented are the mean ($\pm\text{SD}$) of 4 separate determinations measuring the quantity of NAD⁺ reduced in the presence of substrate minus that in the absence of substrate per min per mg protein. As demonstrated in this figure no differences in the total activity of this enzyme could be demonstrated between these two cell populations.

Table 1. Effects of inhibitors of pregnenolone metabolism on the secreted steroid profile of MA-10 LP and the parent population

Sample	ng/(10⁶ cells·h)		
	Pregnenolone	Progesterone	Total Steroid
MA-10 Parent Stock			
+Inhibitors	321.60 ± 8.81	0.00	321.60 ± 8.81
-Inhibitors	13.35 ± 2.70	309.30 ± 71.50	322.65 ± 71.60
MA-10 LP			
+Inhibitors	14.80 ± 1.95	0.00	14.80 ± 1.95
-Inhibitors	7.63 ± 1.20	5.92 ± 1.30	13.55 ± 1.77

Cells were grown in 24-well plates for 24 h in medium containing horse serum, washed twice with PBS⁺ and fresh medium with or without SU-10603 (20 μ M) and cyanoketone (5 μ M) was placed back on cells for 10 min prior to hCG (34 ng/ml) stimulation. After a 1-h incubation, medium was removed and analyzed for pregnenolone and progesterone while trypsin dispersed cells were counted. Means represent the cumulative steroid secreted from four separate experiments and the SEM is the square root of the sum of the squares of the individual SEMs (170). For both clones the pregnenolone secreted in the absence of inhibitors was statistically indistinguishable ($P>0.1$) from the sum of the pregnenolone and progesterone secreted in the absence of inhibitors.

intermediates since the amount of progesterone and pregnenolone synthesized in the absence of inhibitors correlates well with the pregnenolone synthesized in the presence of inhibitors.

Cholesterol Side-Chain Cleavage Activity

Since the lesion which prevents MA-10 LP from secreting as much steroid as the parent population following stimulation was thought to occur prior pregnenolone production we set about to measure the CSCC capacity in the two cell lines. To measure the CSCC capacity of intact cells not limited by the availability of intracellular cholesterol, cells were incubated in the presence of the hydroxylated CSCC intermediate 22R-hydroxycholesterol (22R), which has been demonstrated to serve as an excellent substrate for CSCC in unstimulated cells (124). As illustrated in Table 2 when whole cells from both populations were incubated in the presence of 22R no differences in the side-chain cleavage capacity was demonstrable. As a second measure of the capacity of the CSCC in the two clones, isolated mitochondria were incubated in the presence of 22R with D,L-isocitrate included as a substrate for the generation of reducing equivalents (Table 2). Again no significant differences in the activity of this enzyme complex was evident. Finally, we measured the conversion rate of the true substrate, cholesterol, to pregnenolone and isohexanoic acid by incubating isolated mitochondria in the presence of the reducing equivalent NADPH and [26-¹⁴C]cholesterol as the substrate. In this reaction any cleavage of the radiolabelled cholesterol would result in the liberation of the hydrophilic [¹⁴C]isohexanoic acid and the unlabeled lipophilic pregnenolone. When extracted, the radioactivity present in this water soluble compound will be directly proportional to the cholesterol cleaved by isolated mitochondria. Table 3 demonstrates that this independent assessment of CSCC capacity could detect no differences in the two cell lines.

Based on the amount of the mitochondrial marker enzyme cytochrome c oxidase activity, no differences ($P>0.1$) in the amount of

Table 2. 22R-hydroxycholesterol stimulated side-chain cleavage capacity in parent and MA-10 LP cells and isolated mitochondria

	Pregnenolone Production	
	Whole Cells ng/(10⁶cells·h)	Isolated Mitochondria ng/(mg protein·3h)
Parent Stock	391.5 ± 168.0	3399.5 ± 556.3
MA-10 LP	299.5 ± 38.7	4625.7 ± 554.7

Cells were grown in 24-well plates in medium plus horse serum for 24 h, washed twice with PBS⁺ and medium containing 20 μ M SU-10603 and 5 μ M cyanoketone but lacking horse serum was placed back on cells. After a 10 min preincubation, cultures were stimulated with 50 μ M 22R hydroxycholesterol for 1 h at 37°C, the medium was removed for RIA analysis and trypsin dispersed cells were counted. These data represent the means of four separate observations $\pm$ SD. In these observations no differences ($P>0.1$) in the CSCC capacity were found. Isolated mitochondria from 4 separate cell cultures were incubated in CSCC buffer containing 20 μ M SU-10603 and 5 μ M cyanoketone with or without D,L-isocitrate (10 mM) then stimulated with 50 μ M 22R for 3 h at 37°C prior to the extraction of pregnenolone. In the absence of isocitrate the parent stock and MA-10 LP produced 45.08 ($\pm$ 4.45) and 27.08 ($\pm$ 2.47) ng pregnenolone/mg protein in 3 h, respectively. As in whole cells, there was no difference ($P<0.01$) in pregnenolone synthesized by the mitochondria of the two cell populations.

Table 3. Cholesterol side-chain cleavage activity in isolated mitochondria from parent and MA-10 LP

Clone	Cholesterol Cleaved (nmol/mg protein·2h)
Parent Stock	4.48 ± 0.76
MA-10 LP	3.43 ± 0.56

Isolated mitochondria were incubated in the presence of [26-¹⁴C]cholesterol for 2 h at 37°C in CSCC buffer (see Materials and Methods). The buffer was then removed, uncleaved cholesterol was extracted (125) and the [¹⁴C]isohexanoic acid remaining in the aqueous phase was determined using a Beckman 7800 LSC. The mean ±SD was determined from three individually isolated mitochondrial preparations. Using this method of estimating the capacity of the CSCC no statistically significant differences (P>0.1) were demonstrable between the two cell populations.

mitochondria per cell could be demonstrated between the two cell populations (Fig 6.). Additionally, the amount of mitochondrial protein isolated per million cells was no different between the two cell populations (Fig. 7). In conclusion these data indicate that the lesion in the MA-10 LP subclone which greatly reduces its steroidogenic capacity following stimulation is not due to a decrease in the CSCC capacity or to a decrease in the number of mitochondria in the MA-10 LP clone. Additionally, using the isolation procedures employed in these studies, equal amounts of mitochondrial protein were isolated from each subclone.

cAMP Production

To determine if these cells were able to respond to hCG through the adenylate cyclase system, the hCG-stimulated accumulation of cAMP in the presence of the phosphodiesterase inhibitor IBMX was assessed in the two cell populations over a period of 1 h. As illustrated in Figure 8 the rate of accumulation of cAMP following hCG stimulation was essentially the same in the two cell populations. Although the parent population had a somewhat higher basal level of cAMP and responded to hCG by accumulating more cAMP at 15 min, all other time points examined could detect no significant differences ($P>0.1$) in the accumulation rates between the two cell lines. Perhaps more importantly, no differences in cAMP levels 30 min after hCG stimulation in the absence of IBMX were demonstrable, nor were the levels of cAMP dissimilar in the medium of hCG stimulated cells following a 30-min stimulation (Fig. 9). These data illustrate that the ability of the two cell lines to transduce the trophic hormone stimulation through the adenylate cyclase system is similar and cannot account for the lesion in the low progesterone producing subclone which attenuates its steroidogenic response.

Figure 6. Quantitative analysis of cytochrome C oxidase activity in MA-10 parent stock and MA-10 LP

Twice washed cells were scraped from culture dishes, suspended in Tris-sucrose-EDTA and aliquots removed for cell counts prior to homogenization. Spectrophotometric analysis of cytochrome C oxidase activity was measured in cellular homogenates over 10 min as the decrease in absorption at 500 nm. Data presented are the mean ($\pm$ SD) of four separate determinations. As demonstrated in this figure no difference ($P>0.1$) in the total activity of this enzyme exists between these two cell populations.

Figure 7. Total mitochondrial protein isolated from MA-10 parent stock and MA-10 LP

Twice washed cells were scraped from culture dishes, suspended in tris-sucrose-EDTA and aliquots removed for cell counts prior to homogenization. Cells were homogenized and the mitochondria isolated as described, resuspended in distilled water and aliquots removed for protein analysis. Data presented are the mean $\pm$ SD of four separate cultures of each clone. No difference ($P>0.1$) in the total mitochondrial protein was demonstrable between the two clones.

Figure 8. Total cAMP accumulation in MA-10 parent stock and MA-10 LP following hCG stimulation

Cells were plated at known densities, grown for 24 h in medium containing horse serum at which time they were washed twice and medium lacking serum was placed back on cells. IBMX (0.4 mM) was added to all cultures 10 min prior to hCG stimulation. Cells were processed as described and cAMP levels determined as outlined in the methods. Data shown are the mean $\pm$ SD of four separate determinations.

Figure 9. Total cAMP in MA-10 parent stock and MA-10 LP following hCG stimulation

Cells were plated at known densities, grown for 24 h in medium containing horse serum at which time they were washed twice and medium lacking serum was placed back on cells. Thirty min following hCG stimulation cells and medium were processed as described and cAMP levels determined as outlined in the methods. Data shown are the mean $\pm$ SD of four separate determinations.

Protein Kinase A Activity

Following the activation of adenylate cyclase the cAMP produced binds to the regulatory subunits of the tetrameric protein kinase A (PK-A) which results in the liberation of two activated PK-A monomers. The activated kinase enzymes then phosphorylates other proteins, some of which are enzymes. While the phosphorylation results in the increased activity of some enzymes phosphorylation inhibits the activity of others. It is this pattern of activation and deactivation of cellular proteins which results in the characteristic phenotypic expression of a cell following stimulation with a specific peptide hormone. hCG is thought to activate steroidogenesis through the cAMP second messenger pathway. Since the total amount of hCG stimulated cAMP accumulated in these cells is essentially the same, we have examined the cAMP dependent kinase activity in these clones. Using histone H2B as a non-specific phosphate acceptor and [γ - 32 P] ATP as the phosphate donor, the total PK-A activity present in both cell populations was determined. Cellular homogenates from both clones were incubated in the presence and absence of cAMP and the total TCA precipitable activity determined. As illustrated in Figure 10 no difference exists in the total cAMP independent or the total cAMP dependent PK-A activity between the two clones. While this experimental design does not provide information concerning the endogenous pattern of phosphorylation between the two clones, it does provide evidence that the total cAMP dependent PK-A activity is the same in these cells.

SCP₂ Effects on Isolated Mitochondria

In order to perform studies on isolated mitochondria the structural integrity of the mitochondria following various isolation procedures was examined. This was determined by comparing the steroid production supported by NADPH with that supported by isocitrate under conditions not limited by endogenous cholesterol stores. NADPH, by virtue of its phosphate groups, is unable to cross the mitochondrial membranes and

Figure 10. Total cAMP dependent protein kinase A activity

Following 24 h in culture, cells were washed twice, scraped from the culture dishes and homogenized. Portions of the homogenate were removed for protein analysis while additional aliquots from each clone were incubated at 37°C for 30 min in buffer containing 10 mg/ml histone H2b and [γ -³²P]ATP with or without 1 μ M cAMP. Reactions were terminated by adding an equal volume of ice-cold TCA (20%). Proteins were precipitated for 2 h on ice, captured on glass fiber filters and washed several times with ice-cold TCA (5%). After drying filters were placed into vials, cocktail added and radioactivity assayed. Data shown are the mean $\pm$ SD of four separate determinations.

therefore cannot serve as a source of reducing equivalents for intact mitochondria whereas isocitrate can pass the mitochondrial membranes and generate reducing equivalents by the action of isocitrate dehydrogenase. In these experiments 22R, which can freely cross the mitochondrial membranes and serve as a substrate for the CSCC enzyme complex was used to circumvent the endogenous barriers to cholesterol transport. Figure 11 illustrates that under the isolation conditions used in these experiments NADPH can support no more than 1.8% of the steroid production by isolated mitochondria than can isocitrate indicating that this subcellular fractionation procedure resulted in mitochondrial preparations sufficiently intact to examine the steroidogenic capacity of isolated mitochondria.

To determine whether the lesion in MA-10 LP is associated with SCP₂ the effects of exogenous SCP₂ on mitochondria isolated from control and hCG stimulated cells was examined. Table 4 illustrates that addition of SCP₂ to mitochondria isolated from unstimulated cells was able to increase pregnenolone production 1.35-fold in the parent stock and 1.39-fold in MA-10 LP. Although the fold stimulation reported here was less than the 1.77-fold increase reported by van Noort et al. (62), SCP₂ significantly stimulated steroid production by the mitochondria from both cell populations at $P < 0.005$ and $P < 0.05$ for the parent and MA-10 LP, respectively. Addition of SCP₂ to mitochondria isolated from hCG treated MA-10 LP cells was not, however, able to bring steroid production up to the levels seen in similarly treated parent cells.

Effects of Calcium Inhibitors on Whole Cells

Although it is clear that binding of LH/CG to Leydig cell results in the activation of adenylate cyclase activity, recent studies indicate that hormone stimulation increased cytosolic calcium levels in luteal and rat Leydig cells (99, 131-132). It is also been demonstrated that a rise in intracellular free calcium is required for trophic hormone activation of steroid biosynthesis

Figure 11. Functional integrity of mitochondria isolated from MA-10 parent stock and MA-10 LP

Mitochondria from unstimulated cells were isolated as described and resuspended in CSCC buffer containing 50 μ M 22R, 20 μ M SU-10603 and 5 μ M cyanoketone. To individual mitochondrial samples were added 500 μ M NADPH, 10 μ M isocitrate or no addition (control). Following a 3-h incubation at 37°C reactions were terminated, mitochondria pelleted and pregnenolone measured by RIA directly on aliquots of the supernatant. NADPH could support no more than 1.8% and 0.8% of the steroid production capacity as compared to isocitrate in the parent and MA-10 LP, respectively.

TABLE 4. SCP₂ stimulated steroid production by isolated mitochondria

CELL	TREATMENT	PREGNENOLONE (μ g/mg Mitochondrial Protein)	
Parent	-hCG -SCP ₂	449.72	$\pm$ 25.82
	-hCG +SCP ₂	609.29	$\pm$ 21.79
	+hCG -SCP ₂	2339.27	$\pm$ 155.86
	+hCG +SCP ₂	2704.49	$\pm$ 237.45
MA-10 LP	-hCG -SCP ₂	382.59	$\pm$ 50.58
	-hCG +SCP ₂	531.79	$\pm$ 27.70
	+hCG -SCP ₂	705.15	$\pm$ 106.30
	+hCG +SCP ₂	912.16	$\pm$ 63.62

Cells were incubated in medium lacking serum with or without hCG (34 ng/ml). After 1 h medium was removed and mitochondria were isolated as described and incubated in side-chain cleavage buffer containing isocitrate (10 mM), cyanoketone (5 μ M) and SU-10603 (20 μ M) with or without 1 μ M SCP₂. Following a 30-min incubation, reactions were terminated and pregnenolone assayed by RIA. These data represent the mean pregnenolone secreted by mitochondria isolated from four separate cell cultures $\pm$ SD.

in MA-10 cells (133). While primary cultures have been shown to require exogenous calcium, we have demonstrated that extracellular calcium is not required in the activation of progesterone synthesis by MA-10 cells (data not shown). To examine the role of intracellular free calcium in the activation of whole cell steroidogenesis in these clones cells from both populations were stimulated in the presence or absence of pimozide, which inactivates the calcium-calmodulin complex (134), or TMB-8, which inhibits the release of intracellular calcium (135). Following a 1 h incubation in the presence of hCG, the medium was collected and progesterone measured by RIA. Table 5 illustrates that both TMB-8 and pimozide virtually eliminated the hCG stimulated steroid production by intact cells from both populations. These data indicate that trophic hormone induction of steroidogenesis requires the release of intracellular calcium and the subsequent activation of steroidogenesis following the rise in intracellular free calcium is mediated through the calcium-calmodulin complex in both clones.

Whole Cell Treatment Effects on Mitochondria

Prior data from our laboratory indicated that hCG stimulation resulted in the activation of pregnenolone synthesis in mitochondria isolated from both clones. To investigate the mechanism of this process we first examined the role of calcium in the activation of mitochondrial steroid production. Cells were grown for 24 h, washed twice and medium lacking serum but containing pimozide and TMB-8 was placed back on cells. After a 10-min pre-incubation, hCG (34 ng/ml) was added where indicated and following an additional 1 hour incubation, mitochondria were isolated and incubated in the presence of isocitrate, SU-10603 and cyanoketone for 30 min. Reactions were terminated, mitochondria removed by centrifugation as described and aliquots of the incubation buffer were removed for analysis by RIA. Table 6 illustrates that like whole cells, the cellular activation of pregnenolone production by isolated mitochondria is inhibited by

**Table 5. Effects of inhibitors of intracellular calcium on hCG stimulated
Ssteroid production in whole cells**

Clone	Treatment	ng Pregnenolone/10⁶cells
Parent	Control	4.37 (±0.20)
	hCG	584.96 (±40.92)
	hCG+Pim	8.03 (±0.67)
	hCG+TMB-8	3.41 (±1.20)
MA-10 LP	Control	2.27 (±0.14)
	hCG	33.95 (±3.65)
	hCG+Pim	3.81 (±0.56)
	hCG+TMB-8	4.32 (±0.41)

Cells were incubated for 10 min in medium lacking serum with or without s (100 µM) or TMB-8 (100 µM) prior to addition of hCG (34 ng/ml). After 1 h medium was removed from whole cells and progesterone secretion assessed by RIA. These data represent the mean progesterone secreted of four separate cell cultures ±SD.

Table 6. Effects of inhibitors of intracellular calcium on hCG stimulated steroid production in isolated mitochondria

Clone	Treatment	Isolated Mitochondria (ng/mg protein)
Parent	Control	145.58 (±12.30)
	hCG	475.60 (±18.53)
	hCG+Pim+TMB-8	181.17 (±10.69)
MA-10 LP	Control	53.29 (±6.58)
	hCG	205.23 (±11.69)
	hCG+Pim+TMB-8	78.98 (±4.82)

Cells were incubated for 10 min in medium lacking serum with or without pimozide (100 μ M) and TMB-8 (100 μ M) prior to addition of hCG (34 ng/ml). After 1 h of hormone treatment cells were washed twice with PBS-, mitochondria isolated as described and incubated for 30 min in side-chain cleavage buffer containing isocitrate (10 mM), cyanoketone (5 μ M) and SU-10603 (20 μ M). Reactions were terminated and the secreted pregnenolone was measured by RIA. These data represent the mean pregnenolone secreted by mitochondria isolated from four separate cell cultures $\pm$ SD.

pretreating cells with pimozide and TMB-8. This indicated that intracellular calcium is required in the hCG stimulated activation of steroid synthesis by isolated mitochondria.

Since the stimulation of steroidogenesis by whole cells has been shown to be completely dependent on new protein synthesis, we investigated the role of new protein synthesis in the activation of steroid production by isolated mitochondria. Cells were grown as described above and pre-treated for 10 min with cycloheximide prior to hCG stimulation. Following isolation, the steroidogenic capacity of mitochondria isolated from various treatment groups was assessed. Figure 12 illustrates that the activation of steroid production by isolated mitochondria, like that in whole cells, is completely dependent on de novo protein synthesis.

Mitochondrial Reconstruction Experiments

The observation that hCG stimulated steroid biosynthesis was retained in isolated mitochondria prompted us to examine whether these activated mitochondria could stimulate the steroid production of intact mitochondria isolated from unstimulated parent and MA-10 LP cells. Mitochondria from control and stimulated cells of both the parent and MA-10 LP were isolated and sonicated as described. Sonicate fractions (400 μ l) containing isocitrate, SU-10603 and cyanoketone were pipetted into microfuge tubes and 100 μ l aliquots of intact mitochondria from control and stimulated cells were added and incubated for 30 min at 37°C in a shaking water bath. Reactions were terminated and pregnenolone measured by RIA directly on the supernatant following the removal of mitochondria by centrifugation. Figure 13 illustrates that mitochondrial sonicates from hCG-stimulated parent cells significantly increased steroid production by intact mitochondria isolated from unstimulated parent cells ($P < 0.05$), whereas these sonicate fractions had no effect on intact mitochondria from unstimulated MA-10 LP (Fig. 14). Conversely, sonicate fractions from control or hCG stimulated MA-10 LP cells were without effect on intact

Figure 12. Effects of cycloheximide treatment of whole cells on mitochondria isolated from MA-10 parent stock and MA-10 LP

Following 24 h in culture cells were washed twice and medium lacking serum but containing 1 mM cycloheximide was placed back on cells 10 min prior to hCG (34 ng/ml) stimulation. Following a 1-h incubation mitochondria were isolated as described and incubated in side-chain cleavage buffer containing 10 mM isocitrate, 20 μ M SU-10603 and 5 μ M cyanoketone for 30 min prior to the termination of reactions. Mitochondria were pelleted and the supernatant assayed for pregnenolone by RIA. These data represent the mean $\pm$ SD of four separate cell cultures.

Figure 13. Effects of mitochondrial sonicates from control and stimulated parent and MA-10 LP cells on intact mitochondrial steroid production from unstimulated parent cells

Mitochondrial sonicates were prepared from control and hCG stimulated cells from both cell populations. Following 24 h in culture cells were washed twice and medium lacking serum with or without hCG (34 ng/ml) placed on the cells and incubated for 1 h prior to the isolation and sonication of mitochondria. Intact mitochondria were isolated as described from unstimulated parent cells and incubated in side-chain cleavage buffer containing 10 mM isocitrate, 20 μ M SU-10603, 5 μ M cyanoketone and mitochondrial sonicates for 30 min prior to the termination of reactions. Mitochondria were pelleted and the supernatant assayed for pregnenolone by RIA. These data represent the mean $\pm$ SD of the total pregnenolone in each tube minus the contribution of the sonicated fraction per mg intact mitochondrial protein of four separate cell cultures. While the sonicated fraction from hCG stimulated parent mitochondria (23.6 μ g) significantly increased ($P < 0.01$) pregnenolone production over mitochondrial sonicates from unstimulated parent cells (27.6 μ g), mitochondrial sonicates from control (38.8 μ g) or hCG treated MA-10 LP cells (37.6 μ g) were without effect ($P > 0.1$) on pregnenolone production by intact mitochondria from unstimulated Parent cells.

Figure 14. Effects of mitochondrial sonicates from control and stimulated parent and MA-10 LP cells on intact mitochondrial steroid production from unstimulated MA-10 LP cells

Mitochondrial sonicates were prepared from control and hCG stimulated cells from both cell populations. Following 24 h in culture cells were washed twice and medium lacking serum with or without hCG (34 ng/ml) placed on cells and incubated for 1 h prior to the isolation and sonication of mitochondria. Intact mitochondria were isolated as described from unstimulated MA-10 LP cells and incubated in side-chain cleavage buffer containing 10 mM isocitrate, 20 μ M SU-10603, 5 μ M cyanoketone and mitochondrial sonicates for 30 min prior to the termination of reactions. Mitochondria were pelleted and assayed for pregnenolone by RIA. These data represent the mean $\pm$ SD of the total pregnenolone in each tube minus the contribution of the sonicated fraction per mg intact mitochondrial protein of four separate cell cultures. Sonicated mitochondria from control (27.6 μ g) or hCG stimulated (23.6 μ g) parent cells or from control (38.8 μ g) or hCG stimulated (37.6 μ g) were without effect ($P > 0.1$) on the pregnenolone production by intact mitochondria isolated from unstimulated MA-10 LP cells.

mitochondria from unstimulated parent cells (Fig. 13) or unstimulated MA-10 LP cells (Fig. 14). Since mitochondrial sonicates from the parent but not MA-10 LP can stimulate steroid production in mitochondria isolated from unstimulated parent cells, the dose-dependence of this effect was examined. Figure 15 depicts the effects of increasing amounts of mitochondrial sonicates from hCG stimulated parent and LP cells on intact mitochondria isolated from unstimulated parent cells. This experiment demonstrated that while increasing amounts of LP sonicate had no significant effect on the steroid produced by intact mitochondria isolated from unstimulated parent cells, mitochondrial sonicates from hCG stimulated parent cells exhibited a dose dependent activation of steroid production by these intact mitochondria. Additionally, the lack of a stimulatory effect of MA-10 LP sonicates does not appear to be due to an inhibitor of steroid production since co-incubation of control and stimulated subcellular fractions from MA-10 LP were not able to decrease the stimulatory effect of sonicated mitochondria from stimulated parent cells on intact mitochondria (data not shown).

Mitochondrial Protein Profiles

Studies in our laboratory (89, 136) have indicated that several proteins which are acutely synthesized in response to hCG and Bt₂cAMP appear to be increased in the mitochondrial fraction of the parent cells. This observation coupled with the stimulatory effects of mitochondrial sonicates from stimulated parent cells, but not MA-10 LP, on intact parent mitochondria and the ability of cycloheximide to inhibit the activation of these mitochondria prompted us to examine the protein profiles in these cell populations. Cells were labeled and stimulated as described and 2-D SDS-PAGE was performed on isolated mitochondrial proteins from control, hCG and Bt₂cAMP stimulated cells. Figures 16 and 17 illustrate the profiles obtained from the mitochondria of the parent and MA-10 LP, respectively. As seen in Figure 16A and Figure 17A, the synthesis of proteins depicted as

Figure 15. Dose dependent effects of mitochondrial sonicates from MA-10 parent stock and MA-10 LP on intact parent mitochondria

Mitochondrial sonicates were prepared from hCG stimulated cells from both cell populations. Following 24 h in culture cells were washed twice and medium lacking serum but containing hCG (34 ng/ml) placed on cells and incubated for 1 h prior to the isolation of mitochondria. Intact mitochondria were isolated as described from unstimulated parent cells and incubated in side-chain cleavage buffer containing 10 mM isocitrate, 20 μ M SU-10603, 5 μ M cyanoketone and increasing amount of mitochondrial sonicates for 30 min prior to the termination of reactions. Mitochondria were pelleted and assayed for pregnenolone by RIA. These data represent the mean $\pm$ SD of the total pregnenolone in each tube minus the contribution of the sonicated fraction per mg intact mitochondrial protein of four separate cell cultures.

Figure 16. The effects of hCG and Bt₂cAMP on the synthesis of mitochondrial proteins in MA-10 parent cells

Two million cells were plated into each well of a 6-well tissue culture dish and labelled with 1.0 mCi/ml ³⁵S-methionine-cysteine at 37°C in the presence or absence of stimulation. Cells were collected and mitochondria isolated and prepared for two-dimensional gel electrophoresis as described. The top gel (control) received no stimulation, the middle gel received 1 mM Bt₂cAMP while the lower gel received 34 ng/ml hCG. Proteins 1 through 4 had an apparent molecular weight of 30 kilodalton, protein 5 had an apparent molecular weight of 27 Kd while proteins 6 and 7 were approximately 25 kilodalton. Isoelectric points of these proteins was 6.8, 6.6, 6.3, 6.1, 6.0, 6.0 and 5.8 for proteins 1 through 7, respectively.

Figure 17. The effects of hCG and Bt₂cAMP on the synthesis of mitochondrial proteins in MA-10 LP cells

Two million cells were plated into each well of a 6-well tissue culture dish and labelled with 1.0 mCi/ml ³⁵S-methionine-cysteine at 37°C in the presence or absence of stimulation. Cells were collected and mitochondria isolated and prepared for two-dimensional gel electrophoresis as described. The top gel (control) received no stimulation, the middle gel received 1 mM Bt₂cAMP while the lower gel received 34 ng/ml hCG. Proteins 1 through 4 had an apparent molecular weight of 30 kilodalton, protein 5 had an apparent molecular weight of 27 Kd while proteins 6 and 7 were approximately 25 kilodalton. Isoelectric points of these proteins was 6.8, 6.6, 6.3, 6.1, 6.0, 6.0 and 5.8 for proteins 1 through 7, respectively.

numbers 1-7 were very low or nonexistent in unstimulated cells from either population. Cells stimulated with hCG (Figs. 16B and 17B) resulted in the appearance of proteins 1 and 2. These proteins are 30 kilodaltons in molecular weight and have isoelectric points of 6.8 and 6.6, respectively. As seen in Figures 16C and 17C, cAMP treatment also resulted in the increase of proteins 1 and 2 but cAMP also stimulated the synthesis of proteins 3 and 4. These peptides are also 30 kilodaltons and have isoelectric points of 6.3 and 6.1, respectively.

The appearance of other proteins in response to these treatments are also evident on these gels. In the 25 to 27 kilodalton molecular weight range proteins 5 and 6 can be seen and have isoelectric points of approximately 6.0 while protein number 7 appears in cAMP treated parent cells at pH 5.8 and 25 kilodaltons. These latter proteins are those which we have previously described (89). The quantitation of these proteins shown in Table 7 represent the total integrated intensities of proteins 1-7 using the Visage 2000. Adjustments have been made for differences in loading of gels.

Table 7. Total integrated density of proteins 1 through 7 from parent and MA-10 LP mitochondria

**Parent Mitochondrial Protein Profiles
Total Corrected Integrated Density**

Protein	Control	hCG		cAMP	
	IOD	IOD	(increase)	IOD	(increase)
1	0.636	6.316	(9.9 x)	7.560	(11.9 x)
2	0.358	11.877	(33.2 x)	2.342	(6.5 x)
3	0.000	4.984		2.441	
4	0.469	2.791	(6.0 x)	2.119	(4.5 x)
5	0.000	0.000		0.807	
6	0.000	0.000		0.687	
7	0.000	0.000		0.260	

**MA-10 LP Mitochondrial Protein Profiles
Total Corrected Integrated Density**

Protein	Control	hCG		cAMP	
	IOD	IOD	(increase)	IOD	(increase)
1	0.662	1.608	(2.4 x)	8.580	(12.6 x)
2	0.440	3.735	(5.6 x)	12.505	(18.9 x)
3	2.521	3.640	(1.4 x)	15.652	(6.3 x)
4	1.511	6.072	(4.0 x)	14.873	(9.8 x)
5	0.000	0.000		0.125	
6	0.000	0.497		0.000	
7	0.000	0.775		0.000	

Cells were grown 24 h in culture, washed twice with medium lacking serum and medium lacking serum but containing 1 mCi/ml ³⁵S-cysteine-methionine was placed back on cells prior to addition of hCG (34 ng/ml) or Bt₂cAMP (1 mM). Following a 6-h incubation, mitochondria were collected as described and 200 µl lysis buffer added. Mitochondrial lysates were vortexed 72 h later, the radioactivity of each sample assessed and equal amount of labeled protein subjected to 2-D PAGE as described. The values shown represent the total integrated optical density (IOD) of proteins 1-7 shown in Figs. 16 and 17 as determined with the Visage 2000. These values have been corrected for differences in protein loading on the gels and the fold increase above control of each protein is indicated.

CHAPTER IV

DISCUSSION

In 1963 Ferguson (137) observed that acute stimulation of steroid hormone biosynthesis required the synthesis of new proteins. While in the ensuing 27 years much effort has been dedicated to the characterization of these protein(s) and many new and powerful techniques employed, much about the role of proteins in the regulation of steroidogenesis remains a mystery. Many details concerning cellular events which follow hormonal stimulation have been characterized and numerous acutely stimulated proteins have been described. Neither the function of these protein(s) nor the site of its action have been delineated. In our efforts to understand how acute stimulation of protein synthesis plays a role in the regulation of steroidogenesis in Leydig cells we have employed the MA-10 mouse Leydig tumor cell line. In general, cell cultures offer the advantages of increased material and increased cell purity. While MA-10 cells possess the usual advantages of cell culture systems, they have also retained the highly differentiated phenotypic expression of gonadotropin induced steroid production making them suitable models for studying the regulation of steroidogenesis. We have successfully employed this cell line to describe mitochondrial specific proteins which are induced by hCG and Bt₂cAMP (89, 136). The use of MA-10 cells would seem justified by the finding that Leydig cells from intact rats also synthesize similar mitochondrial specific proteins following hormone treatment (87, 89).

In MA-10 cells it is known that stimulation of steroid production by Leydig cells involves the binding of the trophic hormone LH to specific, high affinity cell surface receptors (106) which activate adenylate cyclase activity through the stimulatory GTP binding protein designated G_s (138), resulting in a rise in intracellular cAMP levels (1, 93). In steroidogenic tissue, hormone stimulation results in the mobilization of intracellular stores of cholesterol, the common steroid precursor, to the inner mitochondrial

membrane for steroid production (139), a process which, in adrenals, requires ongoing protein synthesis (140-141). Additionally, these responses can be mimicked both quantitatively and qualitatively by direct activation of adenylate cyclase as well as by cAMP analogs, indicating the importance of the adenylate cyclase-transducing system in the stimulation of Leydig cell steroid production (111). It is also known that calcium plays a role in the regulation of bovine luteal and rat Leydig cell steroid hormone synthesis (99, 131, 142) although MA-10 cells differ from primary cultures in that MA-10 cells require intracellular stores (143) whereas primary cultures require extracellular calcium in the activation of steroidogenesis (99).

In the initial report of this work we described the isolation of a subclone of the MA-10 Leydig tumor cell line which responded to hCG and Bt₂cAMP by producing much lower levels of progesterone than did the parent stock. The difference in progesterone secretion between the two populations cannot be accounted for by a lesion in the activity of 3 β -HSDH, which converts pregnenolone to progesterone, since no significant accumulation of pregnenolone was demonstrable in control or stimulated MA-10 LP cells. Additionally, quantitative analysis of 3 β -HSDH activity could detect no differences between MA-10 LP and the parent population. Since LH/CG caused a rise in cellular cAMP and Bt₂cAMP could mimic the LH/CG stimulation of steroid production both qualitatively and quantitatively in Leydig cells, the cAMP response was examined in these two cell populations. The total hCG stimulated induction of adenylate cyclase activity was found to be similar in the two cell populations up to one hour after hormone treatment in the presence of a phosphodiesterase inhibitor or 30 min following stimulation in the absence of phosphodiesterase inhibitors. Additionally, no significant secretion of cAMP was apparent in the two cell populations. Taken together, these data indicate that the lesion which reduces the steroidogenic response of MA-10 LP is not due to the inability to transduce the trophic signal via adenylate cyclase. Although the total cAMP is the same in the two cell populations, this approach does not address

whether there is functional coupling of the hCG induced adenylate cyclase to the specific pathways necessary for activation of steroidogenesis or whether regulatory steps subsequent to cAMP generation might be defective.

It has long been known that the rate-limiting enzymatic step in steroid hormone biosynthesis is the side-chain cleavage of cholesterol to yield pregnenolone and isohexanoic acid (84, 144-145). To determine whether the low levels of steroid secreted by MA-10 LP were due to a reduction in the CSCC capacity, steroid production was stimulated with 22R-hydroxycholesterol in whole cells and in isolated mitochondria. This method of measuring the cholesterol side-chain cleavage capacity could detect no differences in the activity of this enzyme complex between the two clones. Furthermore, when isolated mitochondria were incubated in the presence of ^{14}C -labeled cholesterol both clones were able to cleave this substrate into pregnenolone and isohexanoic acid at equal rates. These findings indicated that the inability of MA-10 LP to synthesize steroid following stimulation could not be accounted for by a reduction in the capacity of the CSCC complex. Since the lesion which lowers the ability of MA-10 LP to synthesize hormone following hormone stimulation lies at a point between the generation of cAMP and CSCC capacity, which is precisely the locus believed to be the true rate-limiting step (1), we reasoned that identifying the defect and understanding its mechanism would provide significant insight into the regulation of steroid hormone biosynthesis.

As cholesterol is the common precursor to all steroid hormones, the regulation of its movement within the cell following stimulation is critical to understanding the control of steroid biogenesis. Sterol carrier protein 2 has been reported to regulate cholesterol transport within steroidogenic cells (61, 146-149) and facilitate intramitochondrial movement of cholesterol (150-152). In mitochondria isolated from control and hCG treated cells, SCP₂ was able to stimulate steroid production in the two cell types and this stimulation was of a similar magnitude as that reported for mitochondria from the H540 Leydig cell tumor (62). The inability of SCP₂ to raise steroid

production of mitochondria isolated from hCG treated MA-10 LP cells to the levels seen in similarly treated mitochondria from the parent populations suggests to us that the lesion in MA-10 LP is not due to a defect in this protein. We do not, however, discount the possibility that the lesion in MA-10 LP may be mediated through the interactions of SCP₂ with mitochondrial membranes which may differ between these cells or by some other as yet unknown mechanism.

Mitochondria isolated from hCG stimulated parent cells synthesized four fold more pregnenolone than did mitochondria from untreated cells while stimulation resulted in only a two-fold increase in MA-10 LP mitochondrial steroid production. This indicates that, similar to ACTH stimulated adrenal cells (153-154), the phenotypic expression of increased steroid production is retained in mitochondria isolated from hCG treated MA-10 subclones. Since stimulation of steroid production requires de novo protein synthesis (84, 137) we examined the effects of cycloheximide on the hCG stimulated activation of mitochondrial steroid production. In both cell populations cycloheximide completely prevents the hCG stimulated increase in steroid production by isolated mitochondria. Since cycloheximide treatment is not thought to inhibit delivery of cholesterol to the mitochondria (141, 155) and since the mitochondrial cholesterol content of MA-10 and MA-10 LP is the same (data not shown), this effect is in all likelihood due to an impaired ability on the part of MA-10 LP to move cholesterol from outer to inner mitochondrial membrane, a process believed to require de novo protein synthesis.

Calcium's role in steroidogenesis has been studied in adrenal tissue (156-158), granulosa cells (159), primary cultures of Leydig cells (160) and MA-10 cells (161). It has been demonstrated that calcium is required in the LH induced redistribution of SCP₂ in Leydig cells (61). It is also known that incubation of isolated mitochondria in the presence of 1 mM calcium increased the activity of CSCC although this effect may be attributed to swelling of the mitochondria which can overcome the barrier to endogenous

cholesterol transport. In the present study we have demonstrated that these subclones require the release of intracellular calcium for hCG to stimulate steroid production and the effects of the release of intracellular calcium is mediated through the calcium-calmodulin complex. Additionally, inactivating the calcium-calmodulin complex and preventing the release of intracellular calcium was able to block the activation of mitochondria following hCG stimulation, indicating that calcium mediated events which are required for steroid production are not completely impaired in MA-10 LP since inhibitors of intracellular calcium should not result in inhibition of this pathway if it were already inactive.

Reconstruction experiments were performed to determine whether mitochondria from hCG stimulated cells, which retain their ability to synthesize steroid following isolation, contained a factor or factors which could increase the steroid output of intact mitochondria. Initial studies had demonstrated that hCG stimulated cellular events which resulted in the activation of steroid production by isolated mitochondria from both cell populations. The changes which resulted in activation, however, appeared to be made to a lesser extent in the mitochondria of MA-10 LP since it was observed that their mitochondria produced significantly less steroid than did mitochondria from similarly treated parent cells. These studies demonstrated that factors present in the mitochondria of parent cells following stimulation which resulted in the activation of steroid synthesis can increase steroidogenesis by isolated mitochondria from unstimulated parent cells. Sonicated mitochondria isolated from hCG stimulated parent cells significantly stimulates the synthesis of pregnenolone by intact mitochondria from unstimulated parent cells whereas sonicated mitochondria from hCG stimulated MA-10 LP cells were without effect on these intact mitochondria. These sonicate fractions had no effect on mitochondria isolated from hCG stimulated parent cells indicating that activation of mitochondria was already completed prior to their isolation and no further stimulation with sonicated mitochondrial fractions was

possible. Sonicated mitochondria from hCG stimulated parent or MA-10 LP, however, had no effect on intact mitochondria isolated from MA-10 LP. These data indicated that the activated factor(s) were present in lower concentrations in MA-10 LP or that their mitochondria differed in such a way as to render the factor(s) from the mitochondrial sonicate unable to interact with intact mitochondrial fractions.

To determine whether the inability of mitochondrial sonicates from stimulated MA-10 LP cells was due to a titration effect, a dose-response curve was performed. Increasing concentrations of mitochondrial sonicates isolated from stimulated cells were incubated in the presence of constant amounts of intact mitochondria from unstimulated parent cells and the activation of steroid plotted as a function of the amount of mitochondrial sonicate added. This curve indicated that there is a dose-dependent increase in the steroid output of intact mitochondria by mitochondrial sonicates from hCG stimulated parent cells but not MA-10 LP mitochondrial sonicates. These data indicated that while hCG induces alterations to the mitochondria from both cell populations, these changes do not appear to be as effective in stimulating steroid synthesis in MA-10 LP. Since increasing the amount of sonicated mitochondria added to intact mitochondria still does not increase steroid output in these reconstruction experiments this does not appear to be a question of dosage. The fact that activated mitochondrial sonicates from the parent had no effect on intact MA-10 LP mitochondria suggested that the mitochondria of the MA-10 LP cells may differ in some fundamental way which reduces their capacity to transport cholesterol to the active site of the CSCC complex. One explanation for this observation is that the structure of the MA-10 LP mitochondria may differ in such a way as to make them unable to bind induced proteins necessary for activation of steroid synthesis as well as the parent mitochondria. This could be due to differences in the phospholipid makeup of the mitochondria which could alter the tertiary structure of membrane proteins and render them less effective in moving cholesterol from outer to inner membrane. Kimura and co-workers have

demonstrated that hormone treatment altered the phospholipid composition of adrenal mitochondria (79, 80) and these changes are prevented by cycloheximide treatment (80). Lipid extracts from these activated mitochondria were able to increase pregnenolone synthesis by mitochondria isolated from control cells (162). A diminished capacity of MA-10 LP cells to alter their mitochondrial membrane phospholipid composition could also explain why activated mitochondria from MA-10 LP are without effect in stimulating intact parent mitochondria in these reconstruction experiments.

Previous data indicated that there are proteins which are acutely synthesized following hCG and Bt₂cAMP stimulation and these proteins appear to be associated with the mitochondrial fraction of adrenal cells (163-167), luteal cells (168) and MA-10 cells (89, 136). Similar proteins have been described in the mitochondria of primary rat Leydig cell cultures following stimulation suggesting that these peptides may play a role in the regulation of steroidogenesis (89, 169). Since the activation of mitochondria requires de novo protein synthesis and this activated "factor" is present in the stimulated mitochondria we have performed two-dimensional PAGE profiles of the mitochondrial proteins which are acutely synthesized following hCG and Bt₂cAMP stimulation in these subclones. These profiles demonstrated that Bt₂cAMP stimulated the synthesis of proteins thought to be necessary in the activation of steroid biosynthesis equally in the two clones. Treatment of cells with hCG, however, resulted in the finding that the MA-10 LP cells were less effective in transducing this stimulation and resulted in a decreased synthesis of these proteins. This indicates that the hCG stimulated accumulation of cAMP may not be as tightly coupled to the signal transduction pathway which resulted in the stimulation of steroid synthesis in MA-10 LP as it is in the parent. While this possibility is presently under investigation, it does not explain why Bt₂cAMP, which stimulated the synthesis and accumulation of these proteins in the mitochondrial fraction of both clones, does not result in steroid synthesis of

MA-10 LP equal to that seen in the parent population. To understand this, the role of these proteins in the activation of steroidogenesis as well as the physiological and chemical differences between the mitochondria of the two clones which may affect cholesterol movement within the mitochondria of these cells requires further investigation.

In summary, the lesion which decreases the steroidogenic response of MA-10 LP lies prior to cholesterol side-chain cleavage and does not appear to be due to a defect in SCP₂. hCG stimulation of cellular steroid production appears to involve a complex cellular response. The cell mediated activation of steroid production in isolated mitochondria is blocked by inhibitors of new protein synthesis, requires the release of intracellular calcium and is mediated through the calcium-calmodulin complex in both cell populations. While hCG stimulation results in comparable cAMP generation we do not know whether this response is coupled to the activation of all the pathways which are required for maximal steroid production. We have also demonstrated that hCG induces alterations to the mitochondria of both cell populations which could increase the pregnenolone production of these fractions when incubated in a cell-free system but the activation of the parent mitochondria is much greater than that seen in MA-10 LP. This indicates that the mitochondria of MA-10 LP are either not able to be modified by cellular events which results in activation or the MA-10 LP cells do not mount as effective a cellular regulatory response as do parent cells. Reconstruction experiments indicate that while the mitochondria isolated from hCG stimulated MA-10 LP are themselves activated they do not synthesize as much pregnenolone as the mitochondria from the parent cells and are unable to impart this activating factor to intact mitochondria. This would seem to indicate that the lesion in clone LP results from an inability of trophic hormone treatment to stimulate the necessary alterations in the mitochondria which are required for subsequent steroid production to proceed at the rate seen in the parent population. 2D PAGE protein profile, however, suggest that Bt₂cAMP elicits a comparable rises in some of the

mitochondrial proteins thought to be necessary in the activation of steroidogenesis. These seemingly contradictory ideas suggest to us that the regulation of steroid biosynthesis is a very complex process with multiple regulatory steps all of which must be activated in concert to increase steroidogenesis. Fitting these pieces into a coherent model will require further investigation.

In conclusion, the body of evidence presented in this dissertation indicates that the regulation of steroidogenesis by MA-10 cells takes place at the level of the mitochondria. It is important, therefore, that subsequent studies focus on the steps necessary to activate the mitochondria following isolation in order to understand the regulation of steroid hormone biosynthesis by Leydig cells. The isolation and characterization of these variants have provided a important tool in the study of this process and should continue to be a useful aid in the study of the cellular mechanisms which play a role in the activation of mitochondrial steroid synthesis.

BIBLIOGRAPHY

1. Kilgore, M.W., Stocco, D.M. (1989) *Endocrinology* 124, 1210-1216
2. Kilgore, M.W., Rommerts, F.F.G., Wirtz, K.W.A., Stocco, D.M. (1990) *Mol. Cell. Endocrinol.*, in press
3. Pritchard, J.B. (1955) *Ancient Near East Texts*, 2nd Ed. Princeton University Press, Princeton, NJ
4. Steinach, E. (1940) The cultural aspects of Eunuchism. *Ciba Symposium*, Vol. 8, No. 7, pp. 406-420
5. Berthold, W.J. (1849) *Arch. Anat. Physiol. Wiss. Med.* 16, 42-46
6. Bremener, W.J. (1981) In *The Testis* (Burger, H. and de Kretser, D., eds.) Vol. 1, pp.1-8, Raven Press, New York, NY
7. Bouin, P., Ancel, P. (1903) *Arch. Zool. Exptl. Gen.* 1, 437-523
8. Brown-Séquard, C.E. (1889) *Arch. Phys. Norm. Pathol.* 21, 651-659
9. Russell, L.D., Bartke, A., Goh, J.C. (1989) *Am. J. Anat.* 184, 179-189
10. Sakiyama, R., Pardridge, W.M., Musto, N.A. (1988) *J. Clin. Endocrinol. Metab.* 67, 98-103
11. Schinz, H.R., Slotopolsky, B. (1927) In *Handbuch der Biologischen Arbeitmethoden* (Abderhalden, E., ed.) Vol. 5, sec. 3B, part 4, installment 238
12. Dym, M., Fawcett, D.W. (1970) *Biol. Repro.* 3, 308-326
13. Dym, M. (1973) *Anat. Rec.* 175, 639-656
14. Russell, L.D. (1978) *Anat. Rec.* 190, 99-112
15. Furuya, S., Kumamoto, Y., Sugiyama, S. (1978) *Arch. Androl.* 1, 211-224
16. Castellon, E., Janecki, A., Steinberger, A. (1989) *Mol. Cell. Endocrinol.* 64, 169-175

17. Sun, Y-T., Irby, D.C., Robertson, D.M., De Kretser, D.M. (1989) *Endocrinology* 125, 1000-1010
18. Boccabella, A.V. (1963) *Endocrinology* 72, 787-794
19. Elkington, J.S.H., Blackshaw, A.W.E. (1974) *Aust. J. Biol. Sci.* 27, 47-56
20. Ahmad, N., Haltmeyer, G.C., Eik-Nes, K.B. (1975) *J. Reprod. Fertil.* 44, 107-112
21. Chowdhury, A.K. (1979) *J. Endocrinol.* 82, 331-339
22. Marshall, G.R., Wickings, E.J., Ludecke, D.K., Nieschlag, E. (1983) *J. Clin. Endocrinol. Metab.* 57, 152-161
23. Huang, H.F.S., Marshall, G.R., Rosenberg, R., Nieschlag, E. (1987) *ACTA Endocrinol.* 116, 433-444
24. Hall, P.F. (1988) In *The Physiology of Reproduction* (Knobler, E. and Neill, J.D., eds.), Vol. 1, pp. 975-998, Raven Press, New York, NY
25. Jennes, L., Dalati, B., Conn, P.M. (1988) *Brain Res.* 452, 156-164
26. Dorfman, R.I. (1965) In *Metabolism of Steroid Hormones* (Dorfman, R.I. and Shipley, R.A., eds.), Vol. 1, pp. 410-411, North-Holland Publishers, New York, NY
27. Hooker, C.W. (1948) *Recent Progr. Hormone Res.* 3, 173-188
28. Wattenberg, L.W. (1958) *J. Histochem. Cytochem.* 6, 225-232
29. Levy, H., Deane, H.W., Rubin, B.L. (1959) *Endocrinology* 49, 265-272
30. Christensen, A.K., Mason, N.R. (1965) *Endocrinology* 76, 646-652
31. Dorrington, J.H., Armstrong, D.T. (1975) *Proc. Natl. Acad. Sci. USA* 72, 2677-2679
32. Conn, P.M., Staley, D., Harris, C., Andrews, W.V., Gorospe, W.C., McArdle, C.A., Huckle, W.R., Hansen, J. (1986) *Annul. Rev. Physiol.* 48, 495-555

33. McArdle, C.A., Huckle, W.R., Conn, P.M. (1987) *J. Biol. Chem.* 262, 5028-5035
34. Huckle, W.R., Conn, P.M. (1987) *Endocrinology* 120, 160-169
35. Wooge, C.H., Conn, P.M. (1988) *Mol. Cell. Endocrinol.* 56, 131-140
36. Steinberger, E, Steinberger, A. (1975) In *Handbook of Physiology* (Greep, R.O. and Astwood, E.B., eds.), Vol. 5, pp. 1-46, Raven Press, New York, NY
37. Christensen, A.K. (1975) In *Handbook of Physiology* (Greep, R.O. and Astwood, E.B, eds.), Vol. 5, pp. 57-101, Raven Press, New York, NY
38. Gorospe, W.C., Conn, P.M. (1987) *Endocrinology* 120, 222-229
39. Freeman, D.A., Ascoli, M. (1982) *J. Biol. Chem.* 257, 14231-14238
40. Freeman, D.A., Ascoli, M. (1983) *Biochim. Biophys. Acta* 754, 72-81
41. Miller, J.B., McLean, M.P. (1987) *Biol. Repro.* 36, 729-739
42. Freeman, D.A. (1987) *J. Biol. Chem.* 262, 13061-13068
43. Wessels, N.K., Spooner, B.S., Ash, J.F., Bradley, M.O., Luduena, M.A., Taylor, E.L., Wrenn, J.T., Yamada, K.M. (1971) *Science* 171, 135-138
44. Mrotek, J.J., Hall, P.F. (1975) *Biochem. Biophys. Res. Commun.* 64, 891-899
45. DiBartolomeis, M.J., Jefcoate, C.R. (1984) *J. Biol. Chem.* 259, 10159-10167
46. Martinez, F., Eschegoyen, S., Briones, R., Cuellar, A. (1988) *J. Lipid Res.* 29, 1005-1011
47. Privalle, C.T., Crivello, J.F., Jefcoate, C.R. (1983) *Proc. Natl. Acad. Sci. USA* 80, 701-706

48. Jefcoate, C.R., DiBartolomeis, M.J., Williams, C.A., McNamara, B.C. (1987) *J. Steroid Biochem.* 27, 721-729
49. Privalle, C.T., McNamara, B.C., Dhariwal, M.S., Jefcoate, C.R. (1987) *Mol. Cell. Endocrinol.* 53, 87-101
50. Paphadjopoulos, D., Poste, G., Mayhew, E. (1974) *Biochem. Biophys. Acta* 363, 404-418
51. Gabbiani, G., Charponnier, C., Kohler, L. (1977) *Clin. Exp. Immunol.* 27, 278-288
52. Gabbiani, G., Charponnier, C., Luscher, E.F. (1975) *Proc. Soc. Exp. Biol. Med.* 149, 618-623
53. Gabbiani, G., Charponnier, C., Zumbe, A., Vassalli, P. (1977) *Nature* 269, 697-701
54. Hall, P.F., Charponnier, C., Nakamura, M., Gabbiani, G. (1979) *J. Biol. Chem.* 254, 9080-9086
55. Brinkmann, A.O., Leemborg, F.G., Rommerts, F.F.G., van der Molen, H.J. (1984) *J. Steroid Biochem.* 21, 259-264
56. Chanderbhan, R., Noland, B.J., Scallen, T.J., Vahouny, G.V. (1982) *J. Biol. Chem.* 257, 8928-8934
57. Chanderbhan, R., Kharroubi, A.T., Noland, B.J., Scallen, T.J., Vahouny, G.V. (1986) *Endocrine Res.*, 12, 351-370
58. Scallen, T.J., Noland, B.J., Gavey, K.L., Bass, N.M., Ocker, R.K., Chanderbhan, R., Vahouny, G.V. (1985) *J. Biol. Chem.* 260, 4733-4739
59. Vahouny, G.V., Dennis, P., Chanderbhan, R., Fiskum, G., Noland, B.J., Scallen, T.J. (1984) *Biochem. Biophys. Res. Comm.* 122, 509-515
60. Vahouny, G.V., Chanderbhan, R., Stewart, P., Tombes, R., Keyeyune-Nyombi, E., Fiskum, G., Scallen, T.J. (1985) *Biochim. Biophys. Acta* 834, 324-330

61. van Noort, M., Rommerts, F.F.G., van Amerogen, A., Wirtz, K.W.A. (1988) *Mol. Cell. Endocrinol.* 56, 133-140
62. van Noort, M., Rommerts, F.F.G., van Amerogen, A., Wirtz, K.W.A. (1988) *Biochem. Biophys. Res. Comm.* 154, 60-65
63. Jefcoate, C.R. (1984) *J. Biol. Chem.* 259, 10159-10167
64. Garren, L.D., Gill, G.N., Masui, H., Walton, G.M. (1971) *Recent Prog. Horm. Res.* 27, 433-478
65. Karaboyas, G.C., Koritz, S.B. (1965) *Biochemistry* 4, 462-468
66. Stone, D., Hechter, O. (1954) *Arch. Biochem. Biophys.* 51, 457-469
67. Privalle, C.T., McNamara, B.C., Dhariwal, M.S., Jefcoate, C.R. (1987) *Mol. Cell. Endocrinol.* 53, 87-101
68. Finaz, C., Lefevre, A., Dampfhofer, D. (1987) *Proc. Natl. Acad. Sci. USA* 84, 5750-5755
69. Lambeth, J.D. (1981) *J. Biol. Chem.* 256, 4757-4762
70. Pember, S.O., Powell, G.L., Lambeth, J.D. (1983) *J. Biol. Chem.* 258, 3198-3206
71. Hsu, D., Huang, Y.Y., Kimura, T. (1985) *Endocrine Res.* 11, 45-58
72. Kowluru, R.A., George, R., Jefcoate, C.R. (1983) *J. Biol. Chem.* 258, 8053-8059
73. Hsu, D., Huang, Y.Y., Kimura, T. (1984) *Biochem. Biophys. Res. Commun.* 118, 844-877
74. Kido, T., Kimura, T. (1982) *Endocrinol. Commun.* 9, 9-24
75. Kido, T., Yakamura, F., Kimura, T. (1982) *Biochim. Biophys. Acta* 666, 370-381
76. Kido, T., Kimura, T. (1981) *J. Biol. Chem.* 256, 8561-8568

77. Lambeth, J.D., Kamin, H., Seybert, D.W. (1980) *J. Biol. Chem.* 255, 8282-8288
78. Seybert, D.W., Lancaster, J.R., Lambeth, J.D., Kamin, H. (1979) *J. Biol. Chem.* 254, 12088-12098
79. Igarashi, Y., Kimura, T. (1984) *J. Biol. Chem.* 259, 10745-10753
80. Dimino, M.J., Snitzer, J., Noland, T.A. (1987) *Biol. Repro.* 36, 97-102
81. Igarashi, Y., Kimura, T. (1986) *J. Biol. Chem.* 261, 14118-14124
82. Igarashi, Y., Kimura, T. (1986) *Biochemistry* 25, 6461-6466
83. Kimura, T. (1986) *J. Steroid Biochem.* 25, 711-716
84. Garren, D.L., Ney, R.H., Davis, W.W. (1965) *Proc. Natl. Acad. Sci. USA* 53, 1443-1448
85. Pon, L.A., Orme-Johnson, N.R. (1988) *Endocrinology* 123, 1942-1948
86. Podesta, E.J., Dufau, M.L., Solano, A.R., Catt, K.J. (1978) *J. Biol. Chem.* 253, 8994-9001
87. Pon, L.A., Epstein, L.F., Orme-Johnson, N.R. (1986) *Edocr. Res.* 12, 429-435
88. Pedersen, R.C., Brownie, A.C. (1987) *Science* 236, 188-190
89. Stocco, D.M., Kilgore, M.W. (1988) *Biochem. J.* 249, 95-103
90. Yanagibashi, K., Ohno, Y., Kawamura, M., Hall, P.F. (1988) *Endocrinology* 123, 2075-2082
91. Ascoli, M., Puett, D. (1978) *Proc. Natl. Acad. Sci. USA* 75, 99-102
92. Hunzicker-Dunn, M., Birnbaumer, L. (1985) In *Luteinizing Hormone Action and Receptors* (Ascoli, M., ed.) Vol. 1, pp. 578-132, CRC Press, Inc., Boca Raton, FL
93. Pereira, M.A., Segaloff, D.L., Ascoli, M., Eckstein, F. (1897) *J. Biol. Chem.* 262, 6093-6097

94. Segaloff, D.L., Ascoli, M. (1981) *J. Biol. Chem.* 256, 11420-11423
95. Ascoli, M., Euffa, J., Segaloff, D.L. (1987) *J. Biol. Chem.* 262, 9196-9203
96. Mendelson, C., Dufau, M., Catt, K. (1975) *J. Biol. Chem.* 250, 8818-8823
97. Hall, P.F., Osawa, S., Mrotek, J. (1981) *Endocrinology* 109, 1677-1682
98. Janszen, F.H.A., Cooke, B.A., van Driel, M.J.A., van der Molen, H.J. (1976) *Biochem. J.* 160, 433-437
99. Davis, J.S., Weakland, L.L., Farese, R.V., West, L.A. (1987) *J. Biol. Chem.* 262, 8515-8521
100. Sullivan, M.H.F., Cooke, B.A. (1986) *Biochem. J.* 236, 45-51
101. Eckstein, N., Eshel, A., Eli, Y., Ayalon, D., Naor, Z. (1986) *Mol. Cell. Endocrinol.* 47, 91-98
102. Veldhuis, J.D., Klase, P.A., Demers, L.M., Chafouleas, J.G. (1984) *Endocrinology* 114, 441-448
103. Horflinger, L.J., Albert, P.J., Williams, A.T., Behrman, H.R. (1984) *Endocrinology* 114, 1208-1215
104. Hall, P.F., Osawa, S., Mrotek, J. (1981) *Endocrinology* 109, 1677-1682
105. Pereira, M.A., Segaloff, D.L., Ascoli, M. (1988) *Endocrinology* 122, 2232-2239
106. Ascoli, M. (1981) *Endocrinology* 108, 88-95
107. Buonassisi, V., Sato, G., Cohen, A.I. (1962) *Proc. Natl. Acad. Sci. USA* 48, 1184-1190
108. Pandey, K.N., Inagami, T. (1986) *J. Biol. Chem.* 261, 3934-3939
109. Pandey, K.N., Ascoli, M., Inagami, T. (1985) *Endocrinology* 117, 2120-2127
110. Ascoli, M., Segaloff, D.L. (1987) *Endocrinology* 120, 1161-1169

111. Freeman, D.A., Ascoli, M. (1981) *Proc. Natl. Acad. Sci. USA* 78, 6309-6313
112. Freeman, D.A., Ascoli, M. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7796-7801
113. Pandey, K.N., Inagami, T., Misono, K.S. (1986) *Biochem. Biophys. Res. Commun.* 133, 800-807
114. Finaz, C., Lefevre, A., Dampfhofer, D. (1987) *Mol. Cell. Endocrinol.* 53, 5750-5753
115. Sedmack, J.J., Grossberg, S.F. (1977) *Anal. Biochem.* 79, 544-552
116. Hessling, J.J., Miller, S.A., Levy, N.L. (1980) *J. Immunol. Methods* 38, 315-321
117. Resko, J.A., Norman, R.L., Niswender, G.D., Spies, H.G. (1974) *Endocrinology* 94, 1280-1287
118. Gilman, A.G. (1970) *Proc. Natl. Acad. Sci. USA* 67, 305-311
119. Tovey, K.C., Oldham, K.G., Whelan, J.A.M. (1974) *Clin. Chim. Acta* 56, 221-228
120. Wiebe, J.P. (1976) *Endocrinology* 98, 505-513
121. Stocco, D.M., Hutson, J.C. (1980) *Can. Res.* 40, 1486-1492
122. della-Cioppa, G., Muffly, K.E., Yanagibashi, K., Hall, P.F. (1987) *Mol. Cell. Endocrinol.* 48, 111-120
123. Chaudhary, L.R., Stocco, D.M. (1988) *Biochemie* 70, 1353-1360
124. Brinkman, A.O., Leemborg, F.G., Rommerts, F.F.G., van der Molen, H.J. (1984) *J. Steroid Biochem.* 21, 259-265
125. Bligh, E.G., Dyer, W.J. (1959) *Can. J. Biochem. Physiol.* 37, 911-918
126. Gill, G.N., Walton, G.M. (1979) *Advances in Cyclic Nucleotide Research* 10, 93-106

127. van der Vusse, G.J., Kalkman, M.L., van der Molen, H.J. (1974) *Biocim. Biophys. Acta* 279, 404-414
128. O'Farrell, P.H. (1975) *J. Biol. Chem.* 250, 4007-4021
129. Laemmli, U.K. (1970) *Nature* 227, 680-688
130. Chubb, C., Ewing, L.L. (1979) *Am. J. Physiol.* 237, E239-E246
131. Sullivan, M.H.F., Cooke, B.A. (1986) *Biochem. J.* 236, 45-52
132. Cooke, B.A., Sullivan, M.H.F. (1985) *Mol. Cell. Endocrinol.* 41, 115-122
133. Pereira, M.A., Segaloff, D.L., Ascoli, M. (1988) *Endocrinology* 122, 2232-2239
134. Conn, P.M., Rogers, D.C., Sheffield, T. (1981) *Endocrinology* 109, 1112-1126
135. Yamada, Y., Teraoka, H., Nakazato, Y., Ohga, A. (1988) *Neurosci. Letters* 90, 338-342
136. Stocco, D.M., Chaudhary, L.R. (1990) *Cell. Signal.* in press
137. Ferguson, J.J.Jr. (1963) *J. Biol. Chem.* 238, 2754-2759
138. Birnbaumer, L., Codina, J., Mattera, R., Yatani, A., Scherer, N., Torro, M-J., Brown, A.M. (1987) *Kidney Internatl.* 32, S14-S37
139. Freeman, D.A., Ascoli, M. (1982) *J. Biol. Chem.* 257, 14231-14236
140. Crivello, J.F., Jefcoate, C.R. (1980) *J. Biol. Chem.* 255, 8144-8151
141. Privalle, C.T., McNamara, B.C., Dhariwal, M.S., Jefcoate, C.R. (1987) *Mol. Cell. Endocrinol.* 53, 87-101
142. Cooke, B.A., Sullivan, M.H.F. (1985) *Mol. Cell. Endocrinol.* 41, 115-122
143. Pereira, M.E., Segaloff, D.A., Ascoli, M. (1988) *Endocrinology* 122, 2232-2239

144. Stone, D., Hechter, O. (1954) *Arch. Biochem. Biophys.* 51, 457-462
145. Karaboyas, G.C., Kortiz, S.B. (1965) *Biochemistry* 4, 462-467
146. Chanderbhan, R., Noland, B.J., Scallen, T.J., Vahouny, G.V. (1982) *J. Biol. Chem.* 257, 8928-8934
147. Chanderbhan, R., Khorroubi, A.T., Noland, B.J., Scallen, T.J., Vahouny, G.V. (1986) *Endocrin. Res.* 12, 351-370
148. Tanaka, T., Billheimer, J.T., Strauss III, J.F. (1984) *Endocrinology* 11, 533-540
149. Scallen, T.J., Noland, B.J., Gravey, K.L., Bass, N.M., Ocker, R.K., Chanderbhan, R., Vahouny, G.V. (1985) *J. Biol. Chem.* 26, 4733-4739
150. Conneely, O.M., Headon, D.R., Olson, C.D., Ungar, F., Dempsey, M.E. (1984) *Proc. Natl. Acad. Sci. USA* 81, 2970-2974
151. Vahouny, G.V., Dennis, P., Chanderbhan, R., Fiskum, G., Noland, B.J., Scallen, T.J. (1984) *Biochem. Biophys. Res. Comm.* 122, 509-515
152. Vahouny, G.V., Chanderbhan, R., Stewart, P., Tombes, R., Keyeyune-Nyombi, E., Fiskum, G., Scallen, T.J. (1985) *Biochim. Biophys. Acta* 834, 331-336
153. Jefcoate, C.R., Simpson, E.R., Boyd, G.S. (1974) *Eur. J. Biochem.* 42, 539-551
154. Simpson, E.R., McCarthy, J.L., Peterson, J.A. (1978) *J. Biol. Chem.* 253, 3135-3139
155. Jefcoate, C.R., Dibartolomeis, M.J., Williams, C.A., McNamara (1987) *J. Steroid Biochem.* 27, 721-729
156. Birmingham, M.K., Elliott, F.H., Valère, P.H.L. (1953) *Endocrinology* 53, 687-689
157. Podesta, E.J., Milani, A., Steffen, H., Neher, R. (1980) *Biochem. J.* 186, 391-397

158. Haksar, A. and Péron, F. (1973) *Biochim. Biophys. Acta* 313, 363-371
159. Veldhuis, J.D., Klase, P.A., Demers, L.M., Chafouleas, J.G. (1984) *Endocrinology* 114, 441-449
160. Hall, P.F., Osawa, S., Mrotek, J. (1981) *Endocrinology* 109, 1677-1682
161. Pereira, M.E., Segaloff, D.L., Ascoli, M. (1988) *Endocrinology* 122, 2232-2239
162. Lambeth, J.D., Xu, X.X., Glover, M. (1987) *J. Biol. Chem.* 262, 9181-9188
163. Kruger, R.J., Orme-Johnson, N.R. (1983) *J. Biol. Chem.* 258, 10159-10167
164. Pon, L.A., Orme-Johnson, N.R. (1986) *J. Biol. Chem.* 261, 6594-6599
165. Pon, L.A., Orme-Johnson, N.R. (1988) *Endocrinology* 123, 1942-1948
166. Kruger, R.J., Orme-Johnson, N.R. (1988) *Endocrinology* 122, 1869-1875
167. Alberta, J.A., Epstein, L.F., Pon, L.A., Orme-Johnson, N.R. (1989) *J. Biol. Chem.* 264, 2368-2372
168. Pon, L.A., Orme-Johnson, N.R. (1986) *J. Biol. Chem.* 261, 6594-6599
169. Pon, L.A., Epstein, L.F., Orme-Johnson, N.R. (1986) *Endocrin. Res.* 12, 429-441
170. Howard, P.L., Trainer, T.D. (1980) In *Radionuclides in Clinical Chemistry* (Cawley, L.P., ed.) Vol. 1, pp. 57-104, Little, Brown and Co., Boston, MA