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GONADOTROPIC REGULATION OF  
STEROIDOGENESIS IN RAT  
GRANULOSA CELLS

by

David Frederick Mattice

A thesis submitted to the School of Graduate Studies of the  
University of Ottawa in partial fulfillment of the requirements  
for the degree of Master in Science in the Department of  
Physiology, Faculty of Health Sciences.

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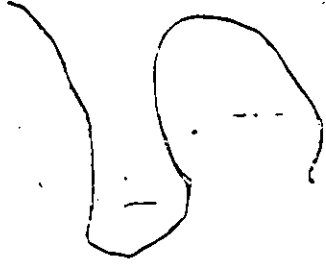
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TABLE OF CONTENTS

	<u>Page</u>
Acknowledgements	1
Abstract	2
I. Introduction	
1. The Ovary	6
2. Stages of Development	6
3. The Steroidogenic Pathway	8
4. The Role of Prostaglandins in Ovarian Steroidogenesis	13a
5. The Role of cyclic AMP and Ca <sup>++</sup> in the action of ACTH on the Adrenal	14
6. The Role of cyclic AMP and Ca <sup>++</sup> in Testicular Steroidogenesis	18
7. Calcium and Granulosa Cell Steroidogenesis	20
8. The Role of Protein Synthesis in Steroidogenesis	23
9. Granulosa Cells as a Model for the Study of Gonadotropic Regulation of Ovarian Steroidogenesis	24
II Rationale and Statement of the Problem	26
III Materials and Methods	29
1. Animals	29
2. Isolation of Granulosa Cells	30
3. Tissue Culture	31
4. Biochemical Assays	31
5. Statistical Analysis	32
6. Experimental Design	32
IV Results	35
1. Stimulation by A23187 of Progesterone Production by Granulosa Cells from Rat Preantral and Proestrous Follicles	35
2. Influence of Various Concentrations of EGTA on Granulosa Cell Progesterone Production Stimulated by A23187	36
3. Influence of FSH and A23187 on Steroidogenesis: Progesterone Production vs. Duration of Tissue Culture	36.
4. Influence of FSH and A23187 on Granulosa Cell Pregnenolone Production	37

	<u>Page</u>
5. Influence of FSH and Calcium on Metabolism of Progesterone	38
6. Involvement of cyclic AMP in the Stimulation of 20 $\alpha$ -OH-P Production by FSH	38
7. Influence of FSH on the Conversion of Exogenous Pregnenolone to Progesterone by Granulosa Cells	39
8. Examination of the Time Course of A23187-Stimulated Conversion of Exogenous Pregnenolone to Progesterone	39
9. Lack of Effect of (Bu) <sub>2</sub> cAMP on the Conversion of Pregnenolone to Progesterone.	40
10. Examination of the inhibition of 3BHSB by Cyanoketone	40
11. Regulation of Progesterone Metabolism to 20 $\alpha$ -OH-P	41
12. Possible Requirement for Protein Synthesis in the Regulation of Protein Production	41
Discussion	43
1. The Regulation of Progestin Production in Rat Granulosa Cells <u>in vitro</u> : Possible Involvement of Calcium and Cyclic AMP	43
2. Calcium - Specific Regulation of 3BHSB in Granulosa Cells	49
3. Gonadotropic Regulation of 3BHSB	51
4. Requirement for Protein Synthesis in the FSH Regulation of Granulosa Cell Progestin Production	53
<u>VI</u> References	57

List of Tables

	<u>Page</u>
1. Influence of FSH and A23187 on progesterone and pregnenolone production.	37a
2. Lack of effect of A23187 on pregnenolone production.	37b
3. Influence of (Bu) <sub>2</sub> cAMP on pregnenolone and progesterone production.	37c
4. Influence of FSH and A23187 on progesterone, 17 $\alpha$ -OH-P, and 20 $\alpha$ -OH-P production.	38a
5. Stimulation of 20 $\alpha$ -OH-P production by A23187.	38b
6. Stimulation of 20 $\alpha$ -OH-P by (Bu) <sub>2</sub> cAMP and Cholera Toxin.	38c
7. Influence of various concentrations of cyanoketone on progestin production.	40a
8. Effect of A23187 and FSH on conversion of exogenous progesterone to 20 $\alpha$ -OH-P.	41a
9. Inability of various concentrations of A23187 to stimulate conversion of exogenous progesterone to 20 $\alpha$ -OH-P.	41b
10. Influence of (Bu) <sub>2</sub> cAMP and Cholera Toxin on the conversion of exogenous progesterone to 20 $\alpha$ -OH-P.	41c
11. Effect of aminoglutethimide phosphate on 20 $\alpha$ -HSD.	41d
12. Effect of different concentrations of cycloheximide on basal progesterone and 20 $\alpha$ -OH-P.	42a
13. Lack of effect of cycloheximide on (Bu) <sub>2</sub> cAMP-stimulated progesterone and 20 $\alpha$ -OH-P production.	42b

List of Figures

	<u>Page</u>
1. Steroidogenic pathways within rat granulosa cells.	8a
2. Stimulation by A23187 of progesterone production and conversion of pregnenolone to progesterone in granulosa cells from PMS-treated rats.	35a
3. Stimulation by A23187 of progesterone production and conversion of pregnenolone to progesterone in granulosa cells from E <sub>2</sub> B-treated rats.	35b
4. Influence of EGTA on A23187-stimulated progesterone production.	36a
5. Time course of progesterone production by granulosa cells in the presence or absence of FSH or A23187.	36b
6. Influence of FSH and A23187 on pregnenolone production in the presence of cyanoketone.	37d
7. Time course of conversion of exogenous pregnenolone to progesterone in the presence or absence of FSH.	39a
8. Concentration-dependent stimulation by FSH of granulosa cell conversion of exogenous pregnenolone to progesterone.	39b
9. Time course of conversion of exogenous pregnenolone to progesterone in the presence or absence of A23187.	39c
10. Effects of cycloheximide on FSH - and A23187 - stimulated progesterone and 20 $\alpha$ -OH-P production.	42c



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## ABSTRACT

The role of calcium and cyclic AMP in the gonadotropic regulation of progesterin production by rat granulosa cells was examined in a chemically-defined medium containing FSH, (Bu)<sub>2</sub>cAMP, cholera toxin, and the calcium ionophore A23187. Both the gonadotropin and A23187 significantly enhanced the production of progesterone and its metabolite, 20 $\alpha$ -hydroxypregn-4-en-3-one (20 $\alpha$ -OH-P) from endogenous steroid substrate but only FSH was effective in stimulating pregnenolone synthesis. The effect of the ionophore appeared to be calcium-related as addition of the calcium chelator, EGTA, to the incubation medium markedly impeded A23187-induced steroid production. Stimulation of progesterone production by calcium was accompanied by an increase in 3 $\beta$ -hydroxysteroid dehydrogenase but not 20 $\alpha$ -hydroxysteroid dehydrogenase activity, as attested by an enhancement of the metabolism of exogenous pregnenolone to progesterone but not of progesterone to 20 $\alpha$ -OH-P. In contrast, while (Bu)<sub>2</sub>cAMP increased pregnenolone and progesterone production, and both (Bu)<sub>2</sub>cAMP and cholera toxin enhanced the metabolism of exogenous progesterone to 20 $\alpha$ -OH-P, the cyclic AMP analogue failed to stimulate the conversion of pregnenolone to progesterone. The stimulation of progesterone production by FSH and A23187 was concentration- and time-dependent. Whereas maximal stimulation of de novo

progesterone synthesis by FSH was evident by 6h (earliest time examined), a significant increase in the conversion of exogenous pregnenolone to progesterone elicited by either the gonadotropin or the ionophore, which also paralleled calcium-stimulated de novo progesterone production, could not be detected until 12h of incubation. Synthesis of new protein appeared to be a part of the mechanism of calcium regulation of progesterone production, as cycloheximide, an inhibitor of protein synthesis, reduced both FSH- and A23187-stimulated progesterone and 20  $\alpha$ -OH-P production by granulosa cells.

In summary, this study indicates that both calcium and cyclic AMP play important but distinct roles in the FSH-stimulation of progesterone production in rat granulosa cells. Whereas the cyclic nucleotide may be involved in the more rapid control of the steroid production via increased synthesis of pregnenolone and/or its metabolism to 20  $\alpha$ -OH-P, calcium participates in the gonadotropic regulation at the level of 3 $\beta$ -hydroxysteroid dehydrogenase via a slower process possibly involving the synthesis of new protein.

## INTRODUCTION

The binding of gonadotropins to plasma membrane receptors of ovarian target cells results in a diversity of cellular responses. One such event is the activation of the membrane-bound adenylate cyclase and subsequent increase of intracellular cyclic adenosine monophosphate (cyclic AMP). Cyclic AMP serves as a second messenger for the extracellular signal, and through a cascade of biochemical reactions elicits an intercellular response. Although many peptide hormones and biogenic amines such as glucagon, follicle-stimulating hormone (FSH), and B-adrenergic catecholamines, stimulate cellular cyclic AMP production (Baxter and Funder, 1979), the specificity of the tropic response to these "primary messengers" is conferred by the presence of specific membrane-bound receptors associated with adenylate cyclase. The cellular response to cyclic AMP is also determined by the type and quantity of protein kinases and phosphorylatable proteins present (Ganong et al., 1983). Three protein components have been demonstrated to be involved in the regulation of cyclic AMP production following hormone receptor interaction. The receptor is located on the surface of the plasma membrane, and may extend through it. The nucleotide regulatory protein (NRP) is situated on the inner

surface of the membrane. Association of the first messenger ligand with its receptor results in the binding of NRP to guanosine triphosphate (GTP). In the presence of  $Mg^{++}$  the NRP-GTP complex activates adenylate cyclase and decreases the affinity of the receptor, facilitating release of the ligand (Catt et al., 1980). This experimental model appears to be applicable for the hormonal activation of cyclic AMP system in many mammalian tissues. In many hormone-dependent systems in which cyclic AMP is a second messenger,  $Ca^{++}$  appears to play a role as a transducing coupler between the hormonal stimulus and the cellular response (Neher, 1982). Changes in the concentration of ionic  $Ca^{++}$  in the cytosol and various intracellular components such as mitochondria, sarcoplasmic or endoplasmic reticulum, may produce the specific physiological signal. Moreover, since the role of the  $Ca^{++}$  ion in many cells is related to, and interdependent on cyclic AMP, a complete understanding of the role of this cyclic nucleotide in gonadotropic stimulation of steroidogenesis requires detailed examination of the action of this divalent cation (Rasmussen and Waisman, 1981).

## The Ovary

The mammalian ovary has two main functions; to produce fertilizable eggs and to synthesize the steroid hormones necessary for menstrual cyclicity, implantation, and maintenance of pregnancy. These functions are under the control of the pituitary gonadotropins, FSH and LH. Menstrual cyclicity is a result of a series of alternating hormonal signals within the hypothalamo-pituitary complex. The steroidogenic cells involved are the theca and granulosa cells of ovarian follicles and luteal cells of corpora lutea formed from follicular cells after ovulation (Birnbaumer and Kirchick, 1983).

## Stages of Development in the Rat Ovary

Cyclic follicular development is a continuum which can be arbitrarily segregated into the following stages: a) primordial follicle, b) preantral follicle, c) antral, mature and pre-ovulatory follicle, d) ovulating and luteinizing follicle, e) corpus luteum, and f) corpus albicans. Mammalian ovaries contain an abundant number of primordial follicles at birth. It is not known why or what stimuli, if any, are responsible for recruitment of particular primordial follicles into the developing pool. Granulosa cells from these follicles contain receptors for FSH but not for LH (Armstrong and Dorrington,

1976, Armstrong et al., 1979; Rani et al., 1981). The theca cells have only LH receptors (Zelevnik et al., 1974). Both cell types can be stimulated by their respective gonadotropins to produce cyclic AMP (Birnbaumer and Kirchick, 1983).

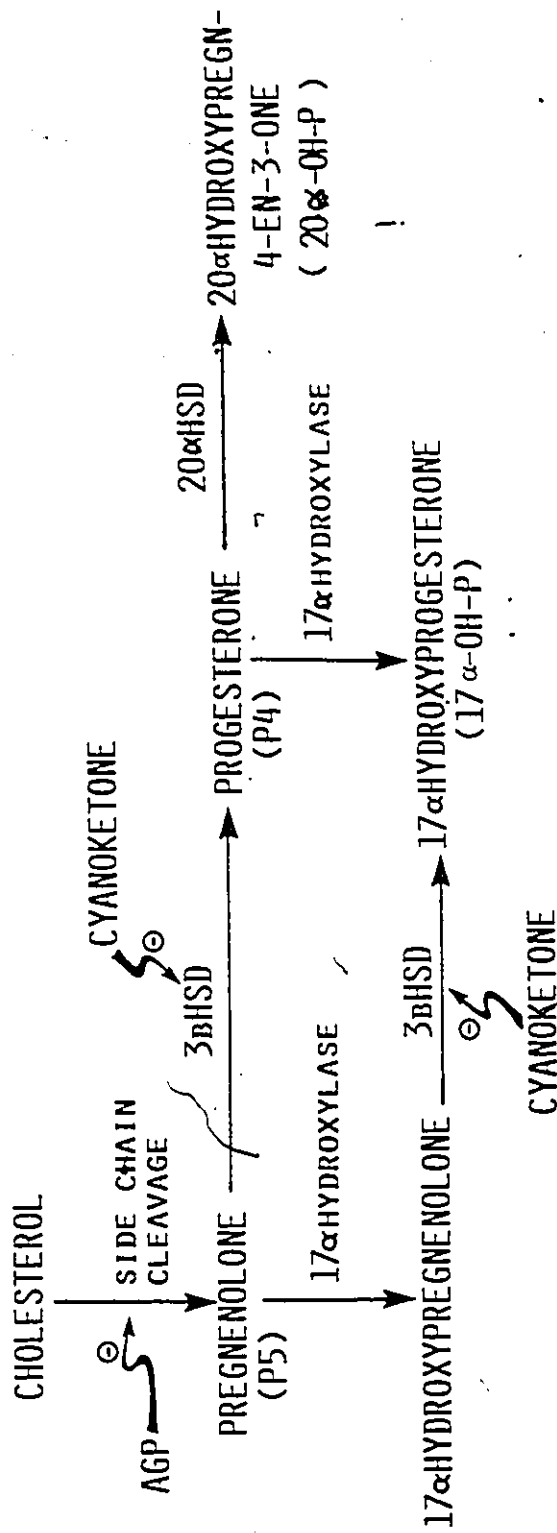
Differentiation of the preantral follicle to the preovulatory follicle requires a delicate interplay among the pituitary hormones FSH, LH, and prolactin (PRL), and theca-granulosa cell(s) interactions. LH stimulates thecal cells to produce androgens. Via a cyclic AMP-dependent mechanism FSH synergizes with androgen from the theca in the induction of receptors for LH and PRL and aromatase activity in granulosa cells (Birnbaumer and Kirchick, 1983). The increase in estradiol production causes proliferation of the theca and granulosa cells in the developing follicles which is accompanied by an enhanced production of proteins and mucopolysaccharides. As the follicle matures PRL and androgen induce the biochemical machinery necessary for progesterone production from cholesterol in the granulosa cells (Wang et al., 1979). LH receptors are present on both theca and granulosa cells and the follicle produces sufficient estradiol to initiate the LH surge and subsequent follicle rupture with expulsion of the mature oocyte (Birnbaumer and Kirchick, 1983).

The LH surge stimulates progesterone production in both theca and granulosa cells. In the theca cells, progesterone is diverted to androgen production. The androgens are then aromatized to estradiol by the granulosa cells (Fortune and Armstrong, 1977; Baird, 1977).

Luteinization involves cellular hypertrophy and differentiation of theca and granulosa cells to luteal cells. This is characterized by a phase of steroidogenic quiescence followed by a reappearance of ability to produce progesterone. In the absence of pituitary PRL, the corpus luteum regresses and cyclicity resumes (Smith et al., 1975; Smith and Neill, 1976). Regression of the corpus luteum occurs with the loss of the glands' ability to produce progesterone with evidence of structural luteolysis. This development of the primordial follicle and the regression of the corpus luteum demands a delicate interplay between pituitary and ovarian hormones.

#### The Steroidogenic Pathway Within the Rat Granulosa Cell

The two most important ovarian steroids, estradiol and progesterone, are produced by the granulosa cells. The granulosa cells are capable of producing progesterone independently of other ovarian cell types. However, the synthesis of estrogen requires a granulosa-theca cell interaction.



STEROIDOGENIC PATHWAYS WITHIN RAT GRANULOSA CELL. INHIBITION OF SIDE CHAIN CLEAVAGE ENZYME AND 3BHSD BY AGP AND CYANOKETONE, RESPECTIVELY


FIGURE 1.

a) Progestin Biosynthesis.

Mature granulosa cells contain all the required enzymes for the de novo synthesis of progesterone (Fig. 1.). Previous studies have demonstrated that both FSH and LH can stimulate de novo progesterone production in granulosa cells (Carnegie and Tsang, 1984; Dorrington and Armstrong, 1979; Richards, 1978). Cholesterol substrate for progesterone production may be derived from either plasma lipoproteins (Gwynne and Strauss, 1982) or de novo cholesterol biosynthesis from acetyl coenzyme A (Wange et al., 1979; Dorrington and Armstrong, 1979b; Norris, 1980). Low density plasma lipoprotein (those having a low lipid:protein ratio) bind to specific plasma membrane "receptors" and the lipoprotein-receptor complex is then internalized by endocytosis (Brown and Goldstein, 1976; Tanaka et al., 1984). The resulting vesicles fuse with lysosomes which hydrolyze the lipoproteins to unesterified cholesterol and amino acids. This rise in unesterified cholesterol inhibits the rate-limiting enzyme in de novo cholesterol synthesis, 3-hydroxy-o-methylglutaryl coenzyme A reductase (HMG-CoA reductase), and stimulates the enzyme responsible for the re-esterification of cholesterol, acyl CoA:cholesterol acyl transferase (ACAT) (Tanaka et al., 1984). Cholesterol esters are stored in lipid droplets and can be hydrolyzed to free cholesterol by stimulation of cholesterol esterase (Behrman and Armstrong, 1969; Heider and Boyett, 1978).

## b) Metabolism of Cholesterol to Pregnenolone

Side-chain cleavage represents the rate-limiting step in the biosynthesis of progestins from cholesterol (Strauss et al., 1982; Henderson et al., 1981). The side-chain hydrolysis of cholesterol to yield the C<sub>21</sub> intermediate, pregnenolone, involves the cytochrome P<sub>450</sub> enzyme located on the inner membrane of mitochondrion. There are two possible modes for regulation of this conversion: 1) increasing substrate availability, or 2) modulation of the P<sub>450</sub> cytochrome activity and level. Henderson et al. (1981) have demonstrated that LH stimulation of luteal cell side-chain cleavage results primarily from increased translocation of cholesterol into the mitochondria, with no detectable increase in mitochondrial P<sub>450</sub> cytochrome concentration. Strauss et al. (1982) established that LH stimulates luteal cell uptake of lipoproteins, presumably by increasing the number of cell surface receptors. They speculated that polyphosphorylated phospholipids serve as intracellular messengers mediating the steroidogenic response to LH. It was also suggested that LH stimulates the synthesis of polyphosphorylated lipid, which is translocated to the mitochondria, resulting in enhanced side-chain cleavage to pregnenolone. Recently, Tanaka et al. (1984) have identified a sterol carrier protein in rat luteal cells similar to the hepatic sterol carrier protein. This hepatic carrier protein



has been demonstrated to stimulate cholesterol transfer and to enhance the activity of membrane bound enzymes involved in cholesterol metabolism, including ACAT (Gavey et al., 1981, Poorthuis and Wirtz, 1982). Toaff et al. (1983) have demonstrated that both FSH and estradiol increase mitochondrial levels of cytochrome P<sub>450</sub> and also stimulate cholesterol side-chain cleavage activity in cultured porcine granulosa cells. Recently Treściak et al. (1986) have demonstrated FSH and (Bu)<sub>2</sub> cAMP induced synthesis of the side-chain cleavage enzymes P<sub>450</sub>, iron-sulfur protein adrenodoxin (SP), and NADPH:ISP reductase (Red) in estrogen-primed rat granulosa cells cultured for 72 hours. Therefore, while it appears that both substrate availability and P<sub>450</sub> cytochrome activity are modes of modulation in many steroidogenic tissues, the regulation of the cholesterol side chain cleavage activity in the rat granulosa cell remains to be clearly defined.

c) Conversion of Pregnenolone to Progesterone

The conversion of pregnenolone to progesterone is a two step process involving the enzymes 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD) and 5- $\alpha$ -3-ketosteroid isomerase ( $\Delta^5, \Delta^4$ -isomerase) (Fevold, 1983). The isomerase is in excess, and its reaction proceeds much faster than that of 3 $\beta$ HSD - the rate limiting step

in this steroid conversion (Philpott and Peron, 1971; Neville and Engel, 1968). 3BHS is primarily located in the smooth endoplasmic reticulum, although some studies have reported its presence in the mitochondria (Fevold, 1983). This enzyme reaction is irreversible and is an important regulatory step in progesterin biosynthesis.

d) Metabolism of Progesterone: 20 $\alpha$ -Hydroxysteroid Dehydrogenase

In the immature rat follicle (preantral), metabolism of progesterone proceeds mainly via 5 $\alpha$ -reduction to primarily 5 $\alpha$ -androstane-3 $\beta$ , 17 $\beta$ -diol. With in vivo administration of pregnant mare's serum gonadotropin (PMSG) and the ensuing follicular maturation, there is a substantial increase in the activity of 20 $\alpha$ -Hydroxysteroid Dehydrogenase (20 $\alpha$ -HSD) (Eckstein and Nimrod, 1979). This enzyme is responsible for the conversion of progesterone to 20 $\alpha$ -hydroxyprogesterone (20 $\alpha$ -OH-P), the major metabolite of progesterone in the adult rat ovary (Ichikawa et al., 1974). Although both FSH and LH have been shown to stimulate 20 $\alpha$ -HSD activity (Eckstein and Nimrod, 1979, Jones and Hseuh, 1981), these observations have been disputed by Moon et al. (1984, 1985).

e) Conversion of Progestins to Androgens: 17 $\alpha$ -Hydroxylase and 17, 20-Lyase.

17 $\alpha$ -hydroxylase catalyzes the conversion of pregnenolone to 17 $\alpha$ -hydroxypregnenolone (17 $\alpha$ -OH-pregnenolone) and progesterone to 17 $\alpha$ -hydroxyprogesterone (17 $\alpha$ -OH-progesterone). 17, 20-lyase converts these two steroids to dehydroepiandrosterone and androstenedione, respectively. While PMSG treatment in vivo has been shown to increase basal activities of these enzymes in granulosa cells, neither are affected by the actions of FSH or LH (Fevold, 1983). In the ovary the theca cells contain high activities of these two enzymes, and in accordance with the "two-cell" theory - first established by Short (1962), progesterone produced by both cell types is converted to androgens within the theca cells. Androgens are then aromatized to estrogens in the granulosa cells (Ryan, 1979; Baird, 1977). This hypothesis is based on the observation that granulosa cells from several species lack the 17 $\alpha$ -hydroxylase and/or 17, 20-lyase enzyme necessary to produce androgens (Armstrong and Dorrington, 1977).

## The Role of Prostaglandins in Ovarian Steroidogenesis

Prostaglandins are present in the ovaries of most animal species and production varies with the functional state of the ovary (Goldberg and Ramwell, 1975). Prostacyclin ( $\text{PGI}_2$ ) has been demonstrated to be the major prostaglandin synthesized in the rat (Poyser and Scott, 1980) and human ovary (Liedkrite and Seifert, 1978). Milvae and Hansel (1980) have demonstrated the ability of  $\text{PGI}_2$  to stimulate progesterone production by bovine corpus luteum both in vivo and in vitro.  $\text{PGI}_2$  has also been shown to stimulate cyclic AMP production in rat granulosa cells (Goff et al., 1978). Further, the secretion of  $\text{PGI}_2$  by rat granulosa cells is increased by LH (Koos and Clark, 1982).  $\text{PGI}_2$  and its stable analogue  $6\text{BPGE}_1$  increase progesterone and estradiol production by granulosa, theca, and corpus luteum cells of the goat (Band et al., 1986). Prostaglandin  $\text{E}_2$  is a potent activator of adenylate cyclase and is known to stimulate ovarian cyclic AMP production. Conversely, prostaglandin  $\text{F}_{2\alpha}$  inhibits adenylate cyclase activity but stimulates cyclic GMP synthesis (Greenspan and Forsham, 1986). Thus it appears that prostaglandins may act as both primary and secondary messengers in the mammalian ovary and have a significant modulatory role in follicular steroidogenesis.

The Role of Cyclic AMP and Ca<sup>++</sup> in the Action of ACTH on the Adrenal.

The involvement of cyclic AMP in the regulation of adrenal steroidogenesis was first reported by Haynes et al. (1959) who demonstrated that adrenocorticotrophic hormone (ACTH) increased the cyclic AMP content of adrenal cortex slices and that exogenous cyclic AMP stimulated corticoid production by rat adrenal glands. Using isolated adrenal cells Beall and Sayers (1972) were able to stimulate steroidogenesis without detectable changes in cyclic AMP production. However, increasing ACTH concentrations evoked parallel increases in both steroid and cyclic AMP production. At the highest concentrations of ACTH employed, cyclic AMP production continued to increase even through steroidogenesis has reached a maximum. These results presented two important points. First, cyclic AMP is not necessarily the sole mediator of ACTH action on steroidogenesis in the adrenal. Second, the stimulation of cyclic AMP production beyond that needed to elicit a supramaximal steroidogenic response by ACTH demonstrates the presence of "spare" receptors, suggesting that an abundance of receptors on the membrane may allow detection of low concentrations of the trophic agent. Similar disparities between cyclic AMP levels and cellular responses have also been observed in adipose tissue, liver, thyroid, and Leydig cells of the testis (Catt and Dufau, 1976).

More recent examination of the mechanism of action of ACTH in isolated adrenocortical cells has led to a better understanding of the biochemical interaction between  $Ca^{++}$  and cyclic AMP in the control of steroidogenesis (Podesta et al., 1979, Podesta et al., 1980, Sola et al., 1979 and Hall et al., 1979). The following simplified scheme has been developed. Once ACTH binds to its membrane receptor adenylate cyclase is activated (as previously described) and catalyzes the conversion of ATP to cyclic AMP. The steady state intracellular level of cyclic AMP is determined by: the rate of formation, and degradation of the cyclic nucleotide, and by its distribution into various intracellular pools. The binding of cyclic AMP to the regulatory subunits of a cyclic AMP-dependent protein kinase results in the dissociation of the catalytic subunits from the holoenzyme, and the phosphorylation of specific preformed protein substrates. It has been suggested that these phosphorylated proteins, and possibly microfilaments and microtubules, by a yet unknown mechanism, might be involved in the translocation of cholesterol across the mitochondrial membrane to the inner matrix. This step is deemed the rate-limiting step of steroidogenic process.

Fakunding et al., (1979) have demonstrated that the steroidogenic responses of isolated adrenal cells to ACTH, angiotensin II, and potassium are dependent on the extracellular concentration of  $Ca^{++}$ .

Physiological concentrations of  $\text{Ca}^{++}$  were necessary to elicit maximal aldosterone responses to all three regulators and steroidogenesis was progressively attenuated by a reduction in extracellular  $\text{Ca}^{++}$  concentration. In contrast, the increase in steroidogenesis elicited by exogenous cyclic AMP was less dependent on extracellular  $\text{Ca}^{++}$ , as evident by its insensitivity to verapamil and lanthanum, two agents known to block cellular uptake of  $\text{Ca}^{++}$  (Singh et al., 1978). Likewise, the stimulatory effect of cyclic nucleotides was unaffected by the presence of the  $\text{Ca}^{++}$  chelating agent, [ethylene-bis(oxyethylenitrilo)] tetraacetic acid (EGTA). Moreover, increase in steroid production induced by cholera toxin, a potent pharmacologic stimulator of adenylate cyclase in steroidogenic tissues via inhibition of GTP hydrolysis (Korval et al. 1974; Haksar et al., 1975), is less sensitive to  $\text{Ca}^{++}$ -deprivation than that by ACTH (Neher, 1982). Hence, the primary requirement for  $\text{Ca}^{++}$  in adrenocortical cells appears to be at the level of hormone receptor-adenylate cyclase coupling since both ACTH binding and steroidogenic stimulation by exogenous cyclic AMP are relatively unaffected in the absence of  $\text{Ca}^{++}$  (Lefkowitz, 1970, Fakunding et al., 1979).

The dependence of ACTH-induced steroidogenesis on extracellular  $\text{Ca}^{++}$  and the ability of  $\text{Ca}^{++}$  to stimulate basal aldosterone production (Shima, 1978) suggests that  $\text{Ca}^{++}$  is either bound or

taken up by the cell. This is supported by the existence of an ACTH-stimulated, cyclic AMP-dependent net accumulation of  $^{45}\text{Ca}^{++}$  in whole adrenal glands in vitro (Leier and Jungman, 1973). A23187 is a monocarboxylic acid antibiotic that promotes  $\text{Ca}^{++}$  movement across cellular membranes. The result is increased cytosolic  $\text{Ca}^{++}$  by both influx of extracellular  $\text{Ca}^{++}$  and release from intracellular stores (Fakunding and Catt, 1982). As observed during B-adrenergic activation of hepatocytes (Assimakopoulos-Jeannet et al., 1977), A23187 has been shown to stimulate phosphatase A and glucose release in a  $\text{Ca}^{++}$ -dependent fashion. Moreover, in the presence of  $\text{Ca}^{++}$ , this ionophore can mimic gonadotropin-releasing hormone effects on LH release by pituitary cells (Conn and Rogers, 1979). Fakunding and Catt (1982) have recently demonstrated that in adrenal glomerulosa cells, the  $\text{Ca}^{++}$  ionophore A23187 increased aldosterone production in a dose-dependent manner. This response was also dependent on the concentration of extracellular  $\text{Ca}^{++}$  and was not accompanied by detectable increases in cyclic AMP. In this context, Lefkowitz et al., (1970) demonstrated that  $\text{Ca}^{++}$  was not necessary for ACTH-receptor interaction.

## The Role of Cyclic AMP and Ca<sup>++</sup> in Testicular Steroidogenesis

Testosterone, the principle hormone produced in the testes, is synthesized from cholesterol in the Leydig cells. 17 $\beta$ -hydroxylase, but not 11- and 21-hydroxylases, is known to be present in these cells. Pregnenolone is hydroxylated in the 17 position and subjected to side chain cleavage to form 17-ketosteroids which are subsequently converted to testosterone. LH stimulates testosterone production in the Leydig cells (Ganong, 1983).

Sandler and Hall (1966) were the first to demonstrate that exogenous cyclic AMP stimulates testicular testosterone production. Using rat testis they observed an increase in both testosterone production and the conversion of [7-<sup>3</sup>H] cholesterol to [<sup>3</sup>H] testosterone. The concentration of cyclic AMP used (0.027 M) was far in excess of physiologic range, but the effect was specific since ATP, ADP, and AMP had no effect at similar concentrations. Connell and Eik-Nes (1968) confirmed this finding in rabbit testis and demonstrated that theophylline, an inhibitor of phosphodiesterase, could stimulate testosterone synthesis at low concentrations (10<sup>-7</sup>M). Studies have also demonstrated that the major effect of LH in Leydig cell testosterone formation is mediated by cyclic AMP and cyclic AMP-dependent protein kinase (Dufau and Catt, 1978, Cooke et al., 1981; Lin, 1985).

The role of  $\text{Ca}^{++}$  in Leydig cell steroidogenesis is not well understood. Basal testosterone production by Leydig cells in vitro has been shown to be unaffected by the absence of  $\text{Ca}^{++}$  in the incubation medium. However, maximal LH-stimulated testosterone formation could only be observed in the presence of the divalent cation. In the absence of  $\text{Ca}^{++}$ , testosterone production was reduced by about seventy percent (Janszen et al., 1976a). Furthermore, verapamil inhibited LH-stimulated testosterone formation (Lin et al., 1979). Evidence has also accumulated in recent years to support the concept that intracellular action of  $\text{Ca}^{++}$  in the testis is mediated through protein phosphorylation (Kimura et al., 1984; Welsh et al., 1980). Lin (1985) has demonstrated the presence of a  $\text{Ca}^{++}$  /phospholipid-dependent protein kinase (kinase C) in rat Leydig cells. Kinase C is strictly dependent on phospholipid, phosphatidylserine, and  $\text{Ca}^{++}$ , and its activity is markedly enhanced by unsaturated diacylglycerol (Kishimoto et al., 1980).

Hall and co-workers (1981) have examined the role of calmodulin in the response of Leydig cells to LH. Trifluoroperazine (TFP), an inhibitor of calmodulin, prevented the stimulation of Leydig cell testosterone production by both LH and cyclic AMP. TFP also inhibited the stimulation of cholesterol transport to mitochondria, as well as attenuating the increase of side-chain cleavage of cholesterol to pregnenolone in isolated mitochondria

elicited by these agents. These observations indicate that both cyclic AMP and  $\text{Ca}^{++}$  are important mediators of gonadotropic stimulation of steroidogenesis in the testis.

#### Calcium and Granulosa Cell Steroidogenesis

While  $\text{Ca}^{++}$  has been shown to be important for maturation of mammalian oocytes (Tsafiriri, 1978) and proteoglycan production by granulosa cells (Lenz et al., 1982), the role of this divalent cation in the gonadotropic regulation of ovarian steroidogenesis is unclear.

Previous studies have shown that omission of  $\text{Ca}^{++}$  from granulosa cell incubations resulted in marked decreases in both progesterone production and aromatization of exogenous testosterone to estradiol in response to gonadotropins (Carnegie and Tsang, 1981; Veldhuis and Klase, 1982). Whereas lanthanum ( $\text{La}^{3+}$ ), a cation known to inhibit cellular  $\text{Ca}^{++}$  uptake and compete with calcium for affinity sites on the plasma membrane (Haksar et al., 1976), markedly reduced FSH-stimulated production of cyclic AMP and progesterone by granulosa cells, basal cyclic AMP production was significantly stimulated by the presence of the trivalent cation (Tsang and Carnegie, 1984; Carnegie and Tsang, 1983). Moreover, inhibition of calmodulin by trifluoroperazine

(TFP) or 1[bis-(p-chlorophenyl)methyl] 3-[2,4-dichloro-B-(2,4-dichlorobenzyl-oxy)-phenethyl]imidazolium chloride (R24571) significantly attenuated cyclic AMP and progesterone production stimulated by FSH, (Bu)<sub>2</sub> cAMP (a cyclic AMP analogue), cholera toxin, Prostaglandin E<sub>2</sub>, or isoproterenol (ISO) (Carnegie and Tsang, 1984; Tsang and Carnegie, 1984). In contrast to the adrenal gland, Ca<sup>++</sup> may be important in the modulation of cellular cyclic AMP levels, possibly via the regulation of adenylate cyclase and phosphodiesterase activity, and thus the gonadotropic control of granulosa cell steroidogenesis (Tsang and Carnegie, 1983, 1984).

Whereas, cobalt and manganese, two inorganic Ca<sup>++</sup> channel blockers, inhibited FSH-stimulated progesterone synthesis, addition of A23187 to granulosa cell culture significantly stimulated the production of the steroid (Tsang and Carnegie, 1984). These findings support the concept that stimulation of uptake of extracellular Ca<sup>++</sup> is an important early event after the binding of the gonadotropin to its receptors and may be an integral part of the activation process through which an appropriate Ca<sup>++</sup> milieu essential for specific biochemical processes may be attained. Moreover, the production of progesterone, but not of cyclic AMP, stimulated by FSH, (Bu)<sub>2</sub> cAMP, cholera toxin, PGE<sub>2</sub>, or ISO was inhibited by the organic Ca<sup>++</sup> entry blocker, verapamil. Dose-response studies with

trifluoroperazine indicated that although both cyclic AMP and progesterone production were reduced in a concentration-dependent manner, low concentrations of this agent were capable of inhibiting steroidogenesis with no significant effect on the net synthesis of the cyclic nucleotide (Tsang and Carnegie, 1984). The hormonal regulation of steroid production by  $\text{Ca}^{++}$  and calmodulin may involve the regulation of protein kinase activity as has been shown in the control of the myosin adenosine triphosphatase (ATPase) system (Hathway and Adelstein, 1979). A  $\text{Ca}^{++}$ -dependent protein kinase has been described in many cellular systems and has been shown to consist of a catalytic subunit and a  $\text{Ca}^{++}$  binding regulatory subunit, subsequently identified as calmodulin. Activation of myosin ATPase may be achieved subsequent to the binding of  $\text{Ca}^{++}$  to the calmodulin subunit, stimulating the phosphorylation of the myosin light chain and facilitating actin-myosin interaction. In this context, Maizels and Jungmann (1982) have demonstrated the presence of  $\text{Ca}^{++}$ /calmodulin-dependent protein phosphorylation activity in the cytosol and nuclear non-histone protein extract of prepubertal rat ovaries and have suggested the possibility of its role in the regulation of ovarian function. Further examination of the  $\text{Ca}^{++}$ -calmodulin system in the granulosa cell will provide a better understanding of the mechanism of action of gonadotropin in this steroidogenic cell. Studies employing EGTA, an agent which readily chelates

extracellular  $Ca^{++}$ , have demonstrated the dependence of progesterone, but not cyclic AMP, production in granulosa cells on extracellular  $Ca^{++}$  (Carnegie and Tsang, 1983; Veldhuis and Klase, 1982). These observations indicate a physiological role of  $Ca^{++}$  both in the control of the adenylate cyclase-cyclic AMP cascade and regulation of biochemical step(s) on the steroidogenic pathway distal to the cyclic AMP cascade. The site(s) of action of  $Ca^{++}$  in the granulosa cell remains to be determined.

#### The Role of Protein Synthesis in the Stimulation of Steroidogenesis

Protein synthesis has been shown to be necessary for steroidogenesis elicited by LH, ACTH, and cyclic AMP. Early work by Hall and Eik-Nes (1962) has demonstrated that inhibitors of protein synthesis, such as puromycin and chloramphenicol, blocked the stimulatory effect of LH on steroidogenesis in rat adrenal slices. The synthesis of cyclic AMP occurred within minutes of adenylate cyclase activation and appeared to be independent of synthesis of new protein (Marsh, 1976; Tanaka and Strauss, 1982).

Farese (1971a) has demonstrated that omission of  $\text{Ca}^{++}$  from the incubation of adrenal slices (stimulated by ACTH) inhibited steroidogenesis and the incorporation of leucine into acid-precipitated protein. This observation is consistent with the concept that protein synthesis required during steroidogenesis may be  $\text{Ca}^{++}$ -dependent. It was suggested that  $\text{Ca}^{++}$  facilitates the transfer of amino acids from aminoacyl tRNA to the elongating peptide chain (Farese, 1971b). Whereas Younglai and Osoko (1981) have shown that the stimulation of steroid production by isolated rabbit ovarian follicles is dependent upon the synthesis of new protein, but not of new RNA. The role of protein synthesis in gonadotropic stimulation of granulosa cell steroid production, and the involvement of  $\text{Ca}^{++}$  are yet to be fully elucidated.

Rat Granulosa Cells: An Invaluable Tool in the Study of  
Gonadotropic Regulation of Ovarian Steroidogenesis

Rat granulosa cells are separated from blood vessels and theca cells by a basement membrane lining the follicle. Granulosa cells are extremely durable, and can easily be isolated by follicle puncture (see Materials and Methods section). A number of in vivo treatments exist for the isolation of granulosa cells at different stages of cell differentiation. Thirty day old rats injected with 17 $\beta$ -estradiol (1 mg/rat; I.P.; days 24 through 29 inclusive) possess ovarian follicles in the preantral stages of

development. Whereas, 30-day-old rats injected with PMSG (4 I.U.;I.P.; Day 28) provide antral or proestrous follicles (Carnegie and Tsang, 1984). These two models offer a convenient means of studying the regulation of granulosa cell steroidogenesis at different stages of development.

There are several advantages of using this cellular model: 1) Immature rats (30 days old) injected as described yield numerous follicles having large numbers of homogeneous cells at the same stage of development. 2) No collagenase or proteolytic enzymes are required for cell isolation, thus avoiding possible changes in cell responsiveness to hormone. 3) Granulosa cells can be cultured in serum-free medium, thus eliminating possible effects of serum factors on steroidogenesis. 4) Primary cultures of granulosa cells, unlike ovarian cell lines, retain hormonal responsiveness and physiological functions (Carnegie and Tsang, 1984).

As in most in vitro experimental models, there are a few disadvantages. The following limitations of granulosa cell culture exist: 1) In vitro preparations are without influence of any possible changes in follicular vasculature or microcirculation which may be important for the delivery of hormones

in vivo (Reynolds, 1973, Niswender et al., 1976; Ellinwood et al., 1978). 2) In vitro culture of isolated granulosa cells eliminates known cell-cell interactions between theca and granulosa cells (Mondschein and Schomber, 1981; Fortune and Armstrong, 1977). 3) The pooling of granulosa cells, and subsequent culture, masks possible structural and functional heterogeneity of granulosa cell subpopulations:

Granulosa cells are arranged in a stratified manner within the follicle with each layer of cells possessing different characteristics. The preovulatory follicle has distinct layers of granulosa cells. The mural layer lines the basement membrane; the cumulus layer surrounds the ovum; and the antral layer lies between these two. The mural cells are likely steroidogenically most active as they possess high levels of 3BHSD, cytochrome P<sub>450</sub> enzyme, and 17 $\alpha$ -hydroxylase (Fevold, 1983). Likewise, the mural granulosa cells contain a much higher concentration of LH receptors than either the antral or cumulus layer (Rajaniemi, 1977; Midgley, 1979). In contrast, the cumulus cells have more PRL receptors than the other two layers (Dunaif et al., 1982). Immunocytochemical studies have demonstrated that the antral granulosa cells have significantly higher intracellular concentrations of cyclic AMP (Dail et al., 1980).

Observation of extensive gap junctions between granulosa cells in vivo indicates the possibility of intercellular communication. Such connections may be important for metabolic exchange between neighbouring cells (Amsterdam, 1976; Bjersing, 1974). Although these cell junctions have been observed in granulosa cell aggregates during in vitro culture (Amsterdam et al., 1981), whether these in vitro situations reflect in vivo conditions remains to be fully elucidated.

Despite the limitations of granulosa cell culture, the in vitro approach has provided invaluable information concerning the endocrine physiology of these cells.

#### Rationale and Statement of Problem

The main objective of this research was to define the site(s) of  $\text{Ca}^{++}$  action in the FSH regulation of steroidogenesis by rat granulosa cells in vitro and examine the role of protein synthesis in the process. More specifically, the following questions were addressed:

- 1) Does  $\text{Ca}^{++}$  regulate the production of pregnenolone from its endogenous precursors, and if so, how does this relate to the action of FSH in stimulating pregnenolone production?

- 2) Is the conversion of pregnenolone to progesterone in granulosa cells regulated by FSH? Does  $\text{Ca}^{++}$  play a role in this regulation?
- 3) Does FSH regulate the metabolism of progesterone? Is  $\text{Ca}^{++}$  involved in this regulation?
- 4) Is the synthesis of new protein(s) involved in these steroidogenic regulations?

## Materials & Methods

### (I) Animals

Immature Sprague - Dawley rats were treated in one of two ways to stimulate follicular growth and development. They were injected intraperitoneally with either 17 $\beta$ -estradiol for 6 days [1 mg/rat in sesame oil, from Day 24 $\pm$ 1 to Day 29 (Armstrong et al., 1979)] and sacrificed on Day 30 $\pm$ 1, or a single dose of 4 IU of pregnant mare's serum gonadotropin (PMSG; Ayerst Labs., Inc., Montreal) on the morning of Day 28 $\pm$ 1 to produce follicles typical of those found during proestrous when harvested on the morning of Day 30 $\pm$ 1 (Fortune and Armstrong, 1977). Ovaries from PMSG-treated rats contained numerous follicles characterized by multiple layers of granulosa and theca cells with an antral cavity present. Ovaries from animals pretreated with 17 $\beta$ -estradiol contained larger numbers of immature preantral follicles with a well-developed granulosa layer, but a thin thecal component.

With the exception of the studies of which the results are indicated in Figure 3, all experiments were performed with granulosa cells from PMSG-treated rats.

## (II) Isolation of Granulosa Cells

Rats were anaesthetized with Halothane b.p. (Superpharm, St. Lambert, Quebec) and under aseptic conditions, the ovaries were removed by vertical laparotomy. With the aid of a dissecting microscope, granulosa cells were collected by puncturing surface follicles with a fine needle and gently squeezing the cells into Eagle's Minimal Essential Medium containing  $\text{NaHCO}_3$  (2.2g/litre), nonessential amino acids (0.1 mM), penicillin-streptomycin (50,000 U/L and 50,000 ug/L, respectively), and fungizone (625 ug/L, all from Grand Island Biological Co., Mississauga, Ontario). The concentration of calcium in the medium was 1.8 mM.

The cells were collected by centrifugation (325 xg, 10 minutes) and resuspended with medium. At this point the cells demonstrated a viability index of 25 - 50% as determined by trypan blue exclusion. In order to increase the viability index, non-viable cells were removed from the preparations by a modification of the method of Farookhi (1982) as indicated hereafter. After the initial cell count, granulosa cells were resuspended to a concentration of  $10^6$  cells/ml and incubated with trypsin (50 ug/ml) for 60 seconds. The reaction was stopped with an addition of excess of soybean trypsin inhibitor (150 ug/ml). The DNA released from dead (lysed) cells was digested with DNase (25 ug/ml) for 5 minutes to prevent clumping

of cells (all three reagents were purchased from Sigma Chemical Co., St. Louis, MO.). The cells were then centrifuged at 320 xg for 10 minutes and resuspended in culture medium. This step was repeated with a subsequent viability check. A viability index of 85 - 100% and a recovery of 60 - 80% of the initial number of viable cells were noted. The cells were then allocated equally to various treatment groups to attain a final concentration of approximately  $3 \times 10^5$  cells/well.

### (III) Tissue Culture

Cells were incubated for 6, 12, or 24 hours in 1 ml of culture medium at 37°C under an atmosphere of 5% CO<sub>2</sub> and 95% air. At the end of culture period, the medium was collected and stored at -20°C pending extraction twice with ether prior to steroid analysis.

### (IV) Biochemical Assays

Medium collected at the end of the culture period was extracted twice with three volumes of diethyl ether which were evaporated under N<sub>2</sub> gas and resuspended in 1 ml of absolute ethanol. Efficiency of the extraction, as determined by simultaneously extracting known amounts of the labelled steroid(s), was 95% - 98%.

The samples were then assayed for pregnenolone, progesterone,  $17\alpha\text{OH-P}$ , and  $20\alpha\text{OH-P}$  content by validated specific radioimmunoassay (Orczyk et al. (1979) and Inaba et al. (1980)). Appropriate aliquots of the standards (0, 5, 10, 20, 40, 80, 160, 320, 640 picograms; in triplicate), unknowns (in duplicate), and interassay pools (for quality control; in duplicate), all dissolved in redistilled ethanol, were pipetted into glass tubes (12 x 75 mm), and evaporated to dryness with  $\text{N}_2$  gas over a warm water bath. They were then resuspended in 100 ul of phosphate-buffered saline in gelatin (PBSG, pH 6.9). One hundred ul (dissolved in PBSG) each of the antisera (at a final concentration which provided 30% binding of the labelled steroid added) and of tritium-labelled steroid (approximately 14,000 cpm) were added. The total incubation volume of the assay was 300 ul. The labelled steroids for the above-mentioned assay were [7 -  $^3\text{H(N)}$ ]-pregnenolone (specific activity, 19.3 Ci/mmole; New England Nuclear (NEN) catalogue #NET-039), [1, 2, 6, 7, 21 -  $^3\text{H(N)}$ ]-progesterone (specific activity, 160 Ci/mmole; NEN catalogue # NET-724),  $17\alpha$ -[1, 2 -  $^3\text{H(N)}$ ]-hydroxyprogesterone (specific activity, 50 Ci/mmole; NEN catalogue # NET-332),  $20\alpha$ -[1, 2 -  $^3\text{H(N)}$ ]-hydroxypregn-4-ene-3-one (specific activity, 53 Ci/mmole; NEN catalogue # NET-236), respectively. Triplicate tubes for determination of the total radioactivity added and of non-specific binding were included. The tubes were gently vortexed and allowed to equilibrate overnight at  $4^\circ\text{C}$ .

On the following day separation of the free and bound steroid was achieved by the addition to each tube (except those for total count determination) of 1 ml of charcoal solution containing Dextran T-70 (25mg/100ml) and alkaline decolorizing charcoal (250mg/100ml). Tubes for total count determination received 1 ml of PBS instead of the charcoal solution. All tubes were immediately vortexed and left in an ice-bath for 15 minutes. The samples were then centrifuged for 15 minutes at 300 xg. The supernatant, which contained the labelled steroid bound to the antibody, was decanted into scintillation vials, and counted in 4.5 ml of scintillation fluid (0.5% PPO in toluene: glacial acetic acid at 27:1) in a beta counter after a minimum of 4 hours.

Steroid levels were determined with the use of the standard curves generated and expressed in picograms per 100,000 cells following correction for extraction loss and interassay variation. The intra- and inter-assay coefficients of variation for the radioimmunoassays were <10% and <20%, respectively.

Antisera used for progesterone and  $20\alpha$ -OH-P assays (kindly donated by Dr. D.T. Armstrong, University of Western Ontario, London) showed negligible cross-reactivity (0.1%) with other progestins, androgens, and estrogens, with the exception of the latter which crossreacted significantly with  $20\beta$ -hydroxypregn-4-en-3-one (8.7%). The antiserum for pregnenolone RIA (kindly donated by Dr. G. Niswender, University of Colorado, Denver) exhibited significant cross-reactivity with  $17\alpha$ -hydroxypregnenolone (12.7%), 5-pregnene-3 $\beta$ ,  $20\alpha$ -diol (25.0%), 5-pregnene-3 $\beta$ ,  $20\beta$ -diol (8.3%), dehydroepiandrosterone (8.7%) and 5-androstene-3 $\beta$ ,  $17\beta$ -diol (5.6%). The  $17\alpha$ -OH-P antiserum (from Dr. D.T. Armstrong) cross-reacted with pregn-4-en- $20\beta$ -ol-3-one (1.2%) but less than 0.1% with progesterone, testosterone, estradiol- $17\beta$ , pregn-4-en- $20\alpha$ -ol-3-one,  $5\alpha$ -pregnane-3,  $20$ -dione and  $5\alpha$ -pregnane-3 $\alpha$ -ol- $20$ -one.

#### (V) Statistical Analysis

Results were analyzed statistically by analysis of variance (ANOVA). The significance of difference between individual treatment groups was determined by the Student t-test.

#### (VI) Experimental Design

##### 1. De Novo Steroid Production

Granulosa cells were incubated for 6, 12, or 24 hours in the absence or presence of FSH (15, 30, 75, or 150 ng/ml;

a highly purified ovine FSH preparation from the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda Maryland, NIAMDD-oFSH-13), A23187 (0.125, 0.25, 0.5, 1.0, 2.0, or 4.0 ug/ml; Sigma), cholera toxin (10ng/ml; Sigma), (Bu)<sub>2</sub> cAMP (0.1 mM; Sigma), and/or EGTA (0-3.6 mM; Eastman Kodak Co, Rochester, New York). In some of the experiments granulosa cells were incubated for 24 hours also in the presence of cyanoketone (0 - 25 mM), a potent inhibitor of 3BHSD, to inhibit metabolism of pregnenolone to progesterone. At the end of the incubation period, medium was collected and frozen for subsequent extraction and analysis of pregnenolone, progesterone, 17 $\alpha$ -OH-P, and 20 $\alpha$ -OH-P. Tissue culture medium containing cells which were not incubated was also analyzed for steroid contents to provide time zero controls.

A23187 (Calbiochem-Behring Corp, San Diego, CA, and Sigma) was suspended in dimethyl sulfoxide (DMSO, Sigma) to form a stock solution of 1.2 mg/ml which henceforth remained shielded from the light. This was divided into 100 ul aliquots and frozen for storage. Control groups received an equal volume of DMSO.

## 2. Conversion of Pregnenolone to Progesterone

The experimental design for these studies was identical to part "1" except that aminoglutethimide phosphate (AGP, 0.75 mM CIBA-Geigi Corporation, Ardsley, Ny.), an inhibitor of side

chain cleavage, and pregnenolone (0.1 uM, Steraloids) were added to the culture media in order to determine the influence of FSH, A23187, (Bu)<sub>2</sub>cAMP, and cholera toxin on the conversion of exogenous pregnenolone to progesterone.

### 3.4 Conversion of Progesterone to 20 $\alpha$ -OH-P

Granulosa cells were incubated for 24 hours in the presence of cyanoketone (25 mM) or AGP (0.75 mM) and progesterone (0.1 mM), with and without FSH (75 ng/ml; NIAMDD-oFSH-13), A23187 (0, 0.25, 0.5, 1.0, 2.0 ug/ml), (Bu)<sub>2</sub>cAMP (0.1 mM), or cholera toxin (10 ng/ml). The ability of the granulosa cell to convert exogenous progesterone to 20 $\alpha$ -OH-P was determined by 20 $\alpha$ -OH-P radioimmunoassay.

### 4. Studies with Cycloheximide, an Inhibitor of Protein Synthesis

Granulosa cells were incubated for 24 hours with various concentrations of cycloheximide (0, 28, 280, 2.8 x 10<sup>4</sup>, and 2.8 x 10<sup>5</sup> ng/ml, Sigma), a translational inhibitor of protein synthesis (Garren et al., 1965; Davis and Garren, 1968) in the absence or presence of FSH (75ng/ml) and A23187 (1.0 ug/ml). At the termination of the culture period the medium was collected for progesterone and 20  $\alpha$  -OH-P radioimmunoassays.

## Results

### 1. Stimulation by A23187 of Progesterone Production by Granulosa Cells from Rat Preantral and Proestrous Follicles.

The influence of A23187 on progesterone production by granulosa cells at different stages of cell differentiation was examined in cultures of granulosa cells isolated from the preantral and pro-estrous ovarian follicles. A23187 stimulated de novo progesterone production and conversion of exogenous pregnenolone to progesterone in a concentration- dependent manner ( $p < 0.01$ ) in both preantral (Fig. 2) and proestrous (Fig. 2) follicles. While a concentration of 4.0 ug/ml of A23187 maximally stimulated de novo progesterone production, this dose was submaximal in the stimulation of pregnenolone to progesterone conversion. However, the amount of progesterone produced from endogenous substrates in response to the ionophore was markedly greater (approx. 3X) by granulosa cells from pro-estrous follicles (Fig. 2) than preantral follicles (Fig. 3). Consequently it was decided that the more differentiated granulosa cell model be used for subsequent studies on the regulation of steroidogenesis.

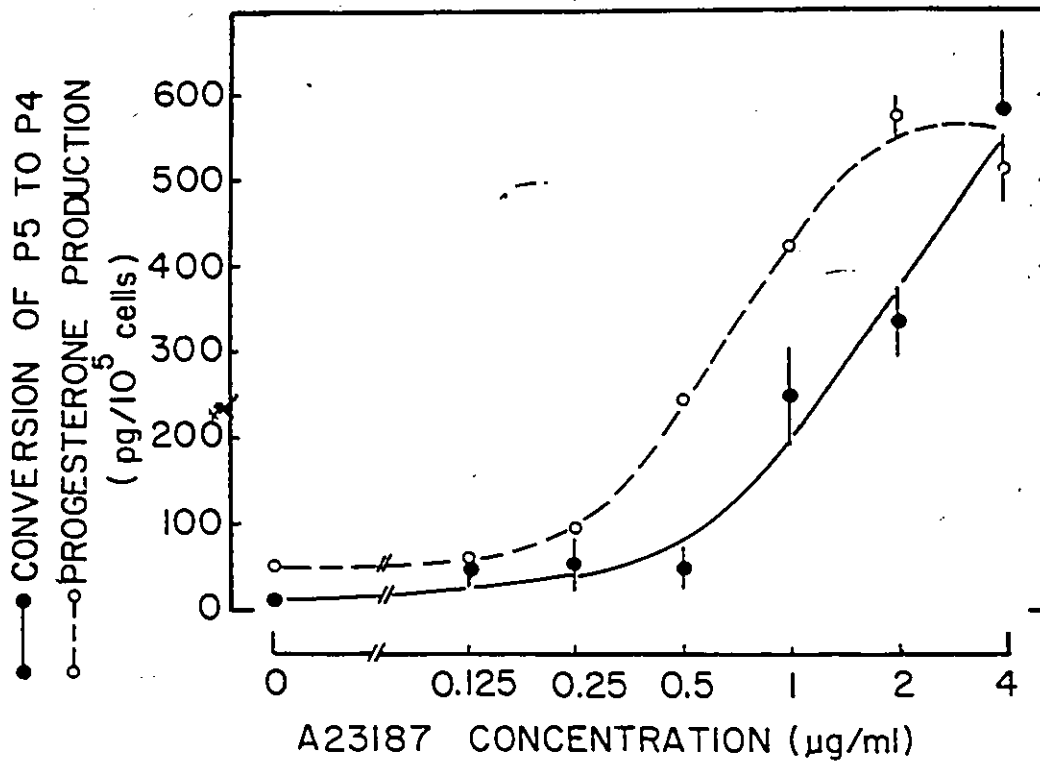


Figure 2.

Stimulation by A23187 of progesterone production and conversion of exogenous pregnenolone (P5, 0.1  $\mu$ m) to progesterone (P4) (in the presence of 0.75 mM AGP) by granulosa cells isolated from PMS-treated rats and incubated for 24 hours. Values are the mean  $\pm$  SEM (n=10 - except 4.0  $\mu$ g/ml: n=8; three experiments).

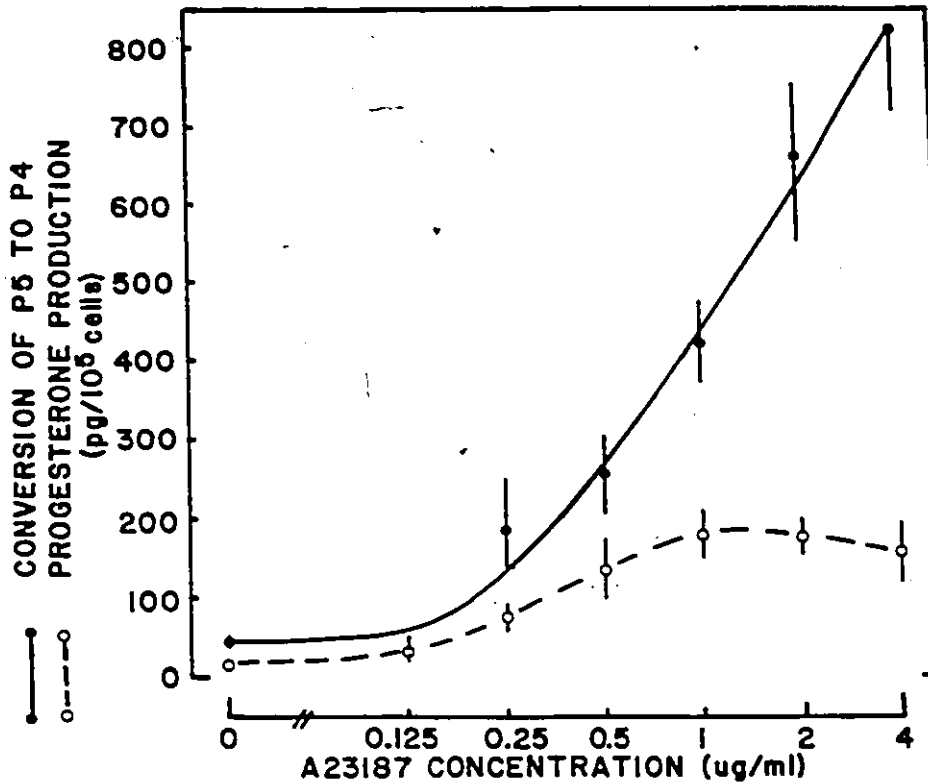


Figure 3.

Stimulation by A23187 of progesterone production and conversion of exogenous pregnenolone (P5, 0.1  $\mu$ M) to progesterone (P4) (in the presence of 0.75 mM AGP) by granulosa cells isolated from  $E_2$ B-treated rats and incubated for 24 hours. Values are the mean  $\pm$  SEM (n=20, five experiments).

2. Influence of Various Concentrations of EGTA on Granulosa  
Cell Progesterone Production Stimulated by A23187.

A23187-stimulated progesterone production appeared to be calcium specific as addition of EGTA to the calcium-containing culture medium resulted in a concentration-dependent ( $P < 0.001$ ) inhibition. Whereas low concentration (0.9 mM) of the calcium chelator elicited a slight but further increase ( $p < 0.01$ ) in progesterone production compared to cultures containing A23187 in the absence of EGTA, concentrations greater than 1.8 mM were inhibitory (Fig. 4).

3. Influence of FSH and A23187 on Steroidogenesis: Progesterone  
Production vs. Duration of Tissue Culture.

A comparison of the mechanism of action of FSH- and calcium-dependent stimulation (as elicited by A23187) of progesterone production was addressed by examining the time course of net progesterone synthesis in the presence or absence of these two agents (Fig. 5). FSH resulted in a significant stimulation (440%,  $p < 0.001$ ) of progesterone production within 6 hours (earliest time examined), whereas an increase (200%,  $p < 0.001$ ) in the production of the steroid by A23187 was not noted until after 12 hours of incubation.

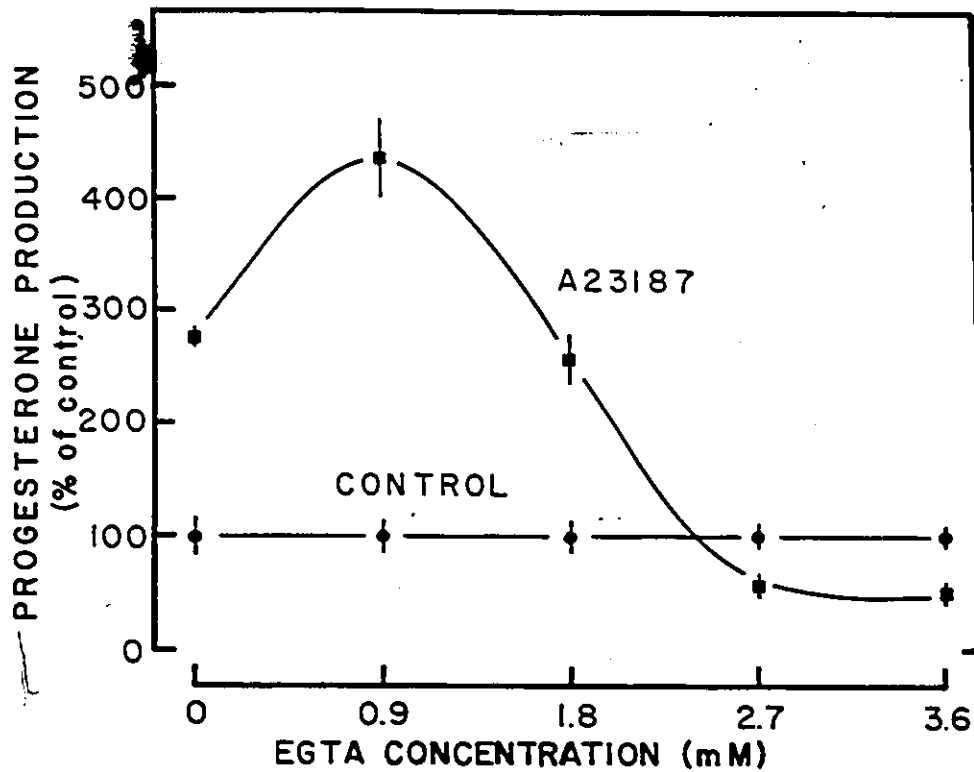


Figure 4.

Influence of EGTA on A23187 (1 ug/ml) - stimulated progesterone production by granulosa cells during a 24h incubation period. The concentration of calcium in the incubation medium was 1.8 mM. Values are the mean  $\pm$  SEM (n=12; three experiments).

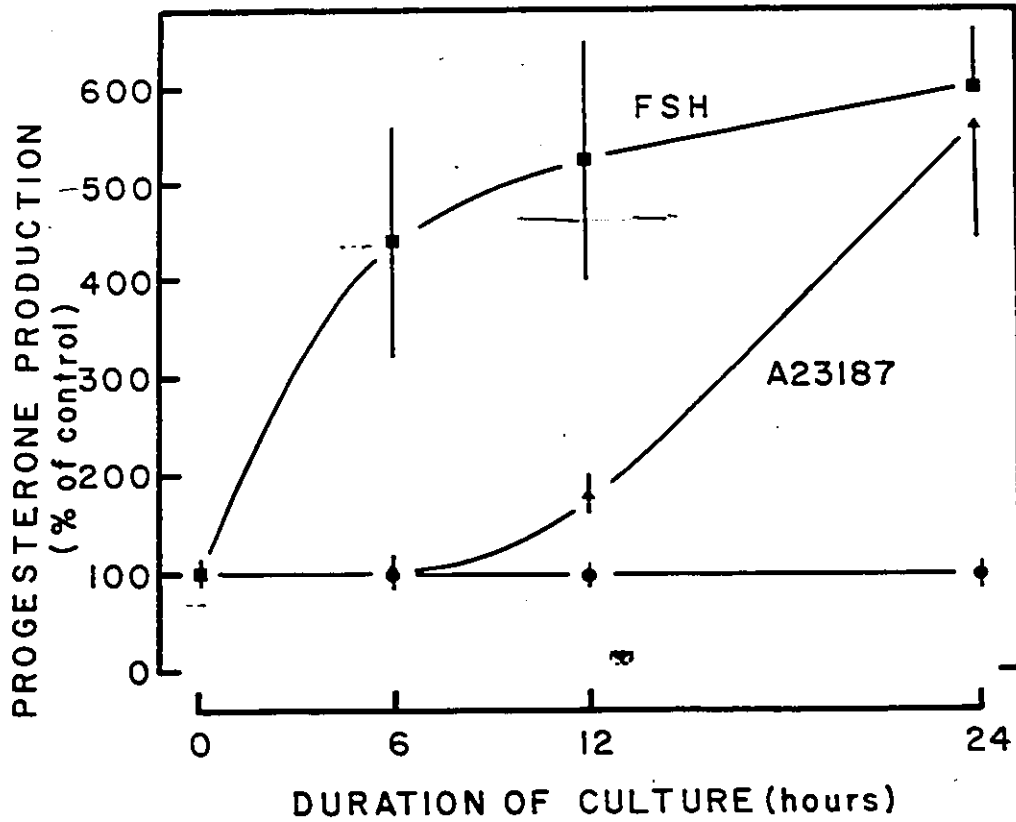


Figure 5.

Time course of progesterone production by granulosa cells incubated in the absence or presence of FSH (75 ng/ml) or A23187 (1 ug/ml). Values are the mean  $\pm$  SEM (n=16; four experiments).

#### 4. Influence of FSH and A23187 in Granulosa Cell Pregnenolone Production.

Table 1 demonstrates the influence of FSH and A23187 on pregnenolone and progesterone production during a 24 h culture period. Whereas FSH (75 ng/ml) stimulated both pregnenolone and progesterone production ( $p < 0.025$  and  $p < 0.005$ , respectively), the calcium ionophore only stimulated progesterone production ( $p < 0.0005$ ) and had no significant effect on pregnenolone production ( $p > 0.05$ ). Further examination with various concentrations of A23187 (0-2.0 ug/ml) also failed to demonstrate a stimulatory action of calcium at this level ( $p > 0.05$ : 1 way ANOVA, table 2). Addition of  $(\text{Bu})_2\text{cAMP}$  (0.1 mM) to the granulosa cell cultures markedly increased the synthesis of both pregnenolone and progesterone (Table 3). The possibility that A23187 indeed stimulated pregnenolone production and that this steroid was rapidly metabolized to progesterone was examined (Fig. 6). In the presence of cyanoketone (25  $\mu\text{M}$ ), an inhibitor of 3 $\beta$ HSD, A23187 also failed to stimulate pregnenolone production ( $p > 0.05$ , Fig. 6). In contrast, FSH elicited a concentration-dependent stimulation of pregnenolone production ( $p < 0.001$ ). Progesterone levels in the media were not substantially increased by either FSH or A23187, an indication of the effectiveness of cyanoketone in preventing the metabolism of pregnenolone to progesterone (Fig. 6).

TABLE 1

Influence of FSH and A23187 on pregnenolone (P5) and progesterone (P4) production (picograms per 105 cells  $\pm$  SEM; n= 12-16; four experiments) by rat granulosa cells cultured for 24 hours.

<u>TREATMENT</u>	<u>P5 (pg/105 cells)</u>	<u>P4 (pg/105 cells)</u>
NONE	21.5 $\pm$ 1.2	42.4 $\pm$ 4.9
FSH (75 ng/ml)	56.0 $\pm$ 8.8	740 $\pm$ 151
A23187 (1.0 ug/ml)	38.0 $\pm$ 21.0	219 $\pm$ 32

TABLE 2

Lack of effect of A23187 on pregnenolone production (pg/105 cells  $\pm$  SEM; n=20; five experiments) by granulosa cells cultured for 24 hours.

<u>TREATMENT</u>	<u>PREGNENOLONE</u>
NONE	53.0 $\pm$ 9.0
A23187 (0.25 ug/ml)	59.6 $\pm$ 8.5
A23187 (0.50 ug/ml)	85.3 $\pm$ 6.8
A23187 (1.0 ug/ml)	59.7 $\pm$ 8.6
A23187 (2.0 ug/ml)	53.9 $\pm$ 7.6

TABLE 3

Influence of (Bu)<sub>2</sub>cAMP on the de novo pregnenolone (P5) and progesterone (P4) production and the conversion of exogenous P5 to P4 (in the presence of 0.75 mM AGP) by granulosa cells cultured for 24h. Values are the mean ± SEM (n=8; two experiments).

EXPT	TREATMENT	STEROID PRODUCED (pg/10 <sup>5</sup> cells)	
		P5	P4
A	NONE	25.7 ± 4.0	76.4 ± 8.7
	(Bu) <sub>2</sub> cAMP (0.1mM)	83.3 ± 5.3	594 ± 37
B	AGP (0.75 mM) ± P5 (0.1 uM)	--	68.8 ± 16.8
	AGP (0.75 mM) ± P5 (0.1 uM)	--	88.8 ± 15.9
	+ (Bu) <sub>2</sub> cAMP (0.1mM)		

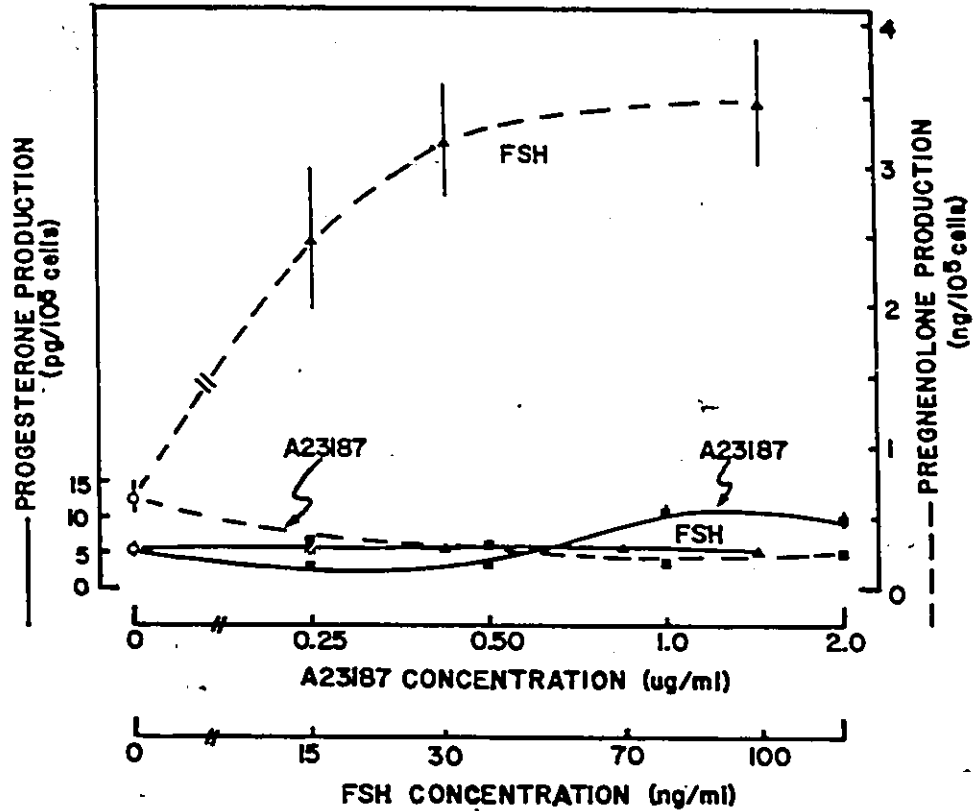


Figure 6.

In the presence of cyanoketone (25 uM), FSH but not A23187 increased granulosa cell pregnenolone production during 24 hour tissue culture. Production of progesterone under these conditions was markedly inhibited. Values are means  $\pm$  SEM (n=12 - except A23187 (2.0 ug/ml): n=8; three experiments).

## 5. Influence of FSH and Calcium on Metabolism of Progesterone

The progesterone metabolites  $20\alpha$ -OH-P and  $17\alpha$ -OH-P were measured in granulosa cell cultures in the presence or absence of FSH or A23187 (Table 4).  $20\alpha$ -OH-P production was stimulated by both FSH ( $p < 0.0005$ ) and A23187 ( $p < 0.0005$ ). In contrast, neither agent had any significant effect on  $17\alpha$ -OH-P synthesis ( $p > 0.05$ ). Further examination with various concentrations of A23187 (0 - 2.0 ug/ml) revealed a concentration-dependent stimulation of  $20\alpha$ -OH-P (Table 4). Maximal stimulation occurred at 1.0 ug/ml, with slight inhibition of the maximal response at 2.0 ug/ml.

## 6. Involvement of Cyclic AMP in the Stimulation of $20\alpha$ -OH-P Production:

To examine the possibility that the mode of action in the stimulation of  $20\alpha$ -OH-P production may be cyclic AMP mediated, the effects of  $(\text{Bu})_2$  cAMP and cholera toxin on  $20\alpha$ -OH-P production by granulosa cells in vitro was studied. Both agents significantly stimulated  $20\alpha$ -OH-P production during a 24 hour culture period ( $p < 0.005$ , Table 6).

TABLE 4

Influence of FSH and A23187 on progesterone (P4), 17 $\alpha$ -OH-P, and 20 $\alpha$ -OH-P production (picograms per 10<sup>5</sup> cells  $\pm$  SEM; n=20; five experiments) by granulosa cells cultured fo 24 hours.

<u>GROUPS</u>	<u>STEROID</u>		
	<u>P4</u>	<u>17<math>\alpha</math>-OH-P</u>	<u>20<math>\alpha</math>-OH-P</u>
CONTROL	44.8 $\pm$ 13	34.7 $\pm$ 6.7	167 $\pm$ 17
FSH (75ng/ml)	554 $\pm$ 118	32.0 $\pm$ 2.7	1297 $\pm$ 189
A23187 (1.0 ug/ml)	223 $\pm$ 45	26.2 $\pm$ 1.4	1603 $\pm$ 299

TABLE 5

Stimulation of 20 $\alpha$ -OH-P production (picograms per 10<sup>5</sup> cells  $\pm$  SEM; n=8; two experiments) by A23187 in granulosa cells cultured for 24h.

<u>GROUPS</u>	<u>20<math>\alpha</math>-OH-P</u>
CONTROL	252 $\pm$ 12
A23187 (0.25 ug/ml)	694 $\pm$ 62
A23187 (0.50 ug/ml)	1284 $\pm$ 108
A23187 (1.0 ug/ml)	1815 $\pm$ 112
A23187 (2.0 ug/ml)	696 $\pm$ 31

TABLE 6

Stimulation of 20 $\alpha$ -OH-P (picograms per 10<sup>5</sup> cells  $\pm$  SEM; n=8; two experiments) by (Bu)<sub>2</sub>cAMP and cholera toxin during a 24-hour granulosa cell culture.

<u>TREATMENT</u>	<u>20<math>\alpha</math>-OH-P</u>
NONE	86.1 $\pm$ 9.2
(Bu) <sub>2</sub> cAMP (0.1 mM)	532 $\pm$ 19
Cholera Toxin (10 ng/ml)	1498 $\pm$ 135

7. Influence of FSH on the Conversion of Exogenous Pregnenolone to Progesterone by Granulosa Cells.

The time course and concentration-dependent effects of FSH on the ability of granulosa cells to metabolize exogenous pregnenolone to progesterone were examined. In contrast to an early stimulation of de novo progesterone production (replotted from Fig. 5) significant gonadotropic stimulation of the conversion of exogenous pregnenolone to progesterone ( $p < 0.001$ ) could not be detected until 12 to 24 hours of culture (Fig. 7). A closer examination of the conversion of pregnenolone to progesterone over a 24 hour period indicated a concentration-dependent enhancement by the gonadotropin ( $p < 0.001$ ). Whereas maximal stimulatory concentrations were observed between 50 and 100 ng/ml a higher concentration (200 ng/ml) appeared to be less effective (Fig. 8).

8. Examination of the Time Course of A23187-stimulated Conversion of Exogenous Pregnenolone to Progesterone.

Figure 9 illustrates the time course of the increase in de novo progesterone production and the capacity to metabolize exogenous pregnenolone to progesterone in granulosa cells cultured in the absence or presence of A23187 (1 ug/ml). In the presence of the

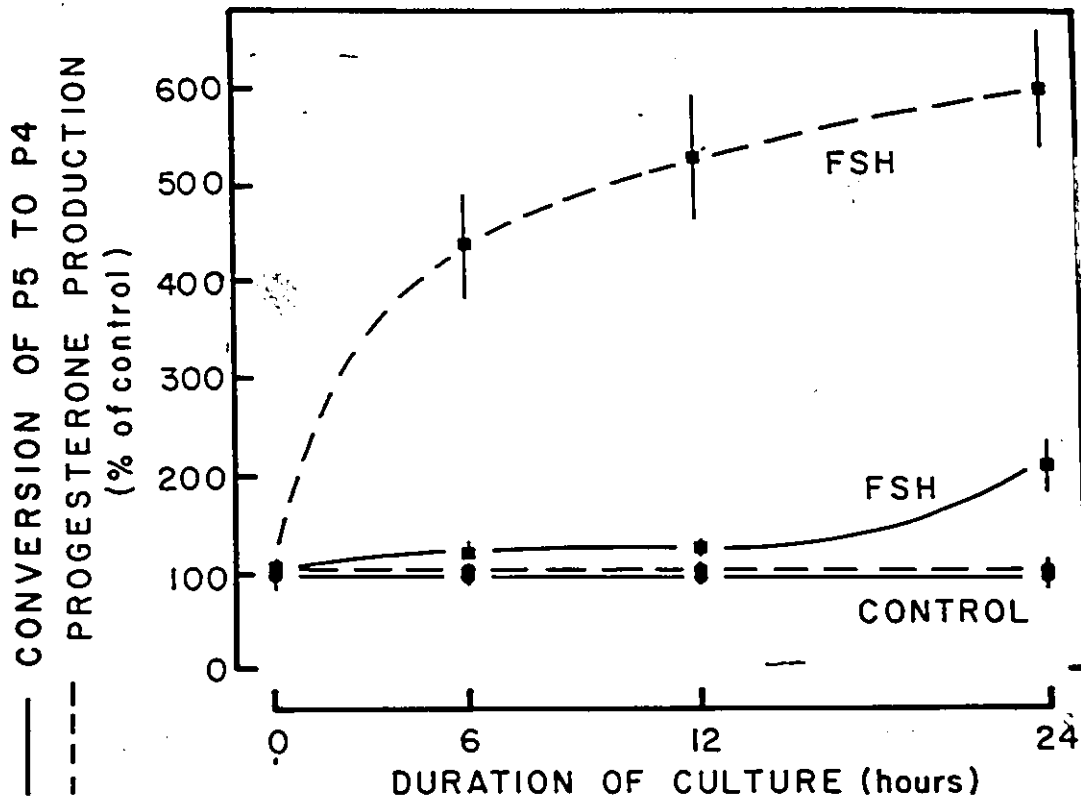


Figure 7.

Time course of progesterone production and conversion of exogenous pregnenolone (P5; 0.1  $\mu$ M) to progesterone (P4) in the presence of AGP (0.75 mM) by granulosa cells incubated for 24 hours in the presence or absence of FSH (75 ng/ml). Values are the mean  $\pm$  SEM (n=16; four experiments).

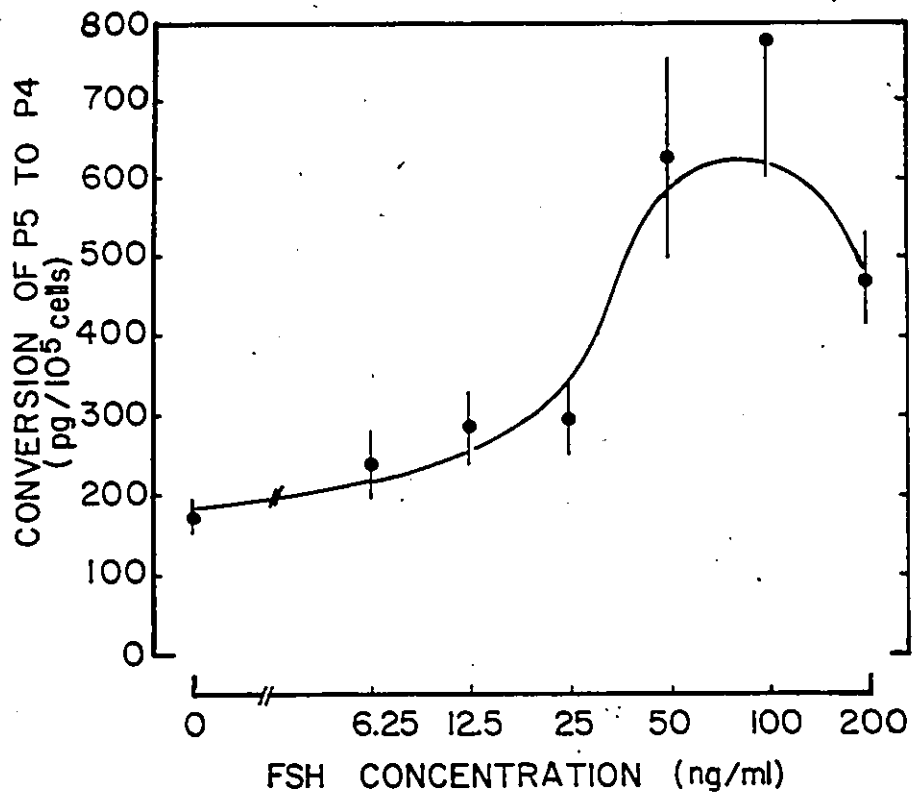


Figure 8.

Concentration-dependent stimulation by FSH of granulosa cell conversion of exogenous pregnenolone (P5; 0.1  $\mu$ M) to progesterone (P4) during a 24 hour culture period. Medium contained AGP (0.75 mM) to inhibit production of endogenous pregnenolone. Values are means  $\pm$  SEM (n=12; three experiments).

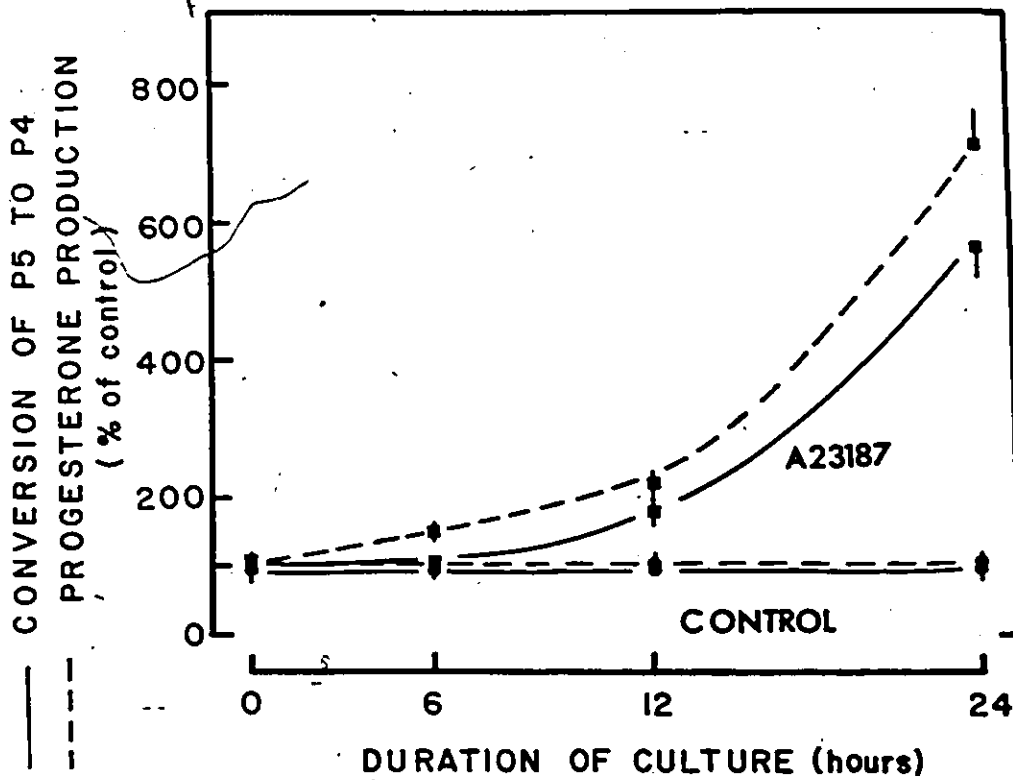


Figure 9.

Time course of progesterone production and conversion of exogenous pregnenolone (P5; 0.1  $\mu$ M) to progesterone (P4) in the presence of AGP (0.75 mM) by granulosa cells incubated in the presence or absence of A23187 (1  $\mu$ g/ml). Values are the mean  $\pm$  SEM (n=16; four experiments).

ionophore, the time-dependent increase in the conversion of pregnenolone to progesterone paralleled that of progesterone production. As with de novo progesterone production, significant stimulation by A23187 of the conversion of pregnenolone to progesterone could not be detected until 12 to 24 hours of culture ( $p < 0.001$ ).

9. Lack of Effect of  $(\text{Bu})_2$  cAMP on the Conversion of Pregnenolone to Progesterone.

Addition of the cyclic AMP analogue,  $(\text{Bu})_2$  cAMP, at a concentration which markedly stimulated pregnenolone, progesterone and  $20\alpha\text{-OH-P}$  production (0.1 mM; Table 6 & 13), failed to significantly affect ( $P > 0.05$ ) the conversion of exogenous pregnenolone to progesterone in a 24-hour culture period (Table 3B).

10. Examination of the Inhibition of 3BHSB by Cyanoketone.

Granulosa cells, cultured for 24 hours in the presence of different concentrations of cyanoketone produced 50 to 70 times more pregnenolone than those cultured in the absence of the inhibitor. Production of progesterone was markedly attenuated ( $p < 0.001$ ; Table 7). Cyanoketone had no significant effect on production of  $20\alpha\text{-OH-P}$  ( $p > 0.05$ ).

TABLE 7

Influence of various concentrations of cyanoketone (CYK) on progestin production in 24 hour cultures. Values are means  $\pm$  SEM (n=8; two experiments).

TREATMENT	STEROID PRODUCTION (pg/10 <sup>5</sup> cells)		
	PREGNENOLONE	PROGESTERONE	20 $\alpha$ -OH-P
1. NONE	46.5 $\pm$ 4.3	213 $\pm$ 40	614 $\pm$ 167
2. CYK (25 $\mu$ M)	2691 $\pm$ 563	41.1 $\pm$ 9.3	846 $\pm$ 249
3. CYK (50 $\mu$ M)	2267 $\pm$ 426	43.3 $\pm$ 12.7	614 $\pm$ 124
4. CYK (100 $\mu$ M)	3429 $\pm$ 685	44.0 $\pm$ 16.4	1023 $\pm$ 248

## 11. Regulation of Progesterone Metabolism to 20 $\alpha$ -OH-P.

In the presence of cyanoketone, an agent added to the culture medium to block endogenous production of progesterone, FSH significantly stimulated the conversion of exogenous progesterone to its major metabolite, 20 $\alpha$ -OH-P ( $p < 0.0005$ ) (Table 8). Addition of A23187 to the culture (1.0 ug/ml) failed to elicit a significant effect. Various concentrations of A23187 (0 - 2.0 ug/ml) also had no stimulatory action on this conversion ( $p > 0.05$ ). High concentrations of the ionophore (1 - 2 ug/ml) appeared to be inhibitory ( $p < 0.005$ ; Table 9). In contrast, both (Bu)<sub>2</sub>cAMP (0.1 mM) and cholera toxin (10 ng/ml) significantly stimulated the metabolism of progesterone to 20 $\alpha$ -OH-P ( $p < 0.0005$ , Table 10). When AGP (0.75 mM) was added to the granulosa cell culture to block the progestin pathway at the cholesterol side chain step, neither (Bu)<sub>2</sub>cAMP, cholera toxin, nor FSH stimulated conversion of exogenous progesterone to 20 $\alpha$ -OH-P (Table 11,  $p > 0.05$ ). However, while AGP attenuated the cyclic AMP-mediated stimulation of 20 $\alpha$ HSD, the ability of progesterone to serve as substrate was unaffected.

TABLE 8

Influence of A23187 and FSH in vitro on conversion of exogenous progesterone (P4; 0.1  $\mu$ M) to 20 $\alpha$ -OH-P (in the presence of cyanoketone (25  $\mu$ M)) by granulosa cells in 24 hours.

Values are means  $\pm$  SEM (n=8; two experiments).

<u>TREATMENT</u>	<u>20<math>\alpha</math>-OH-P (pg/10<sup>5</sup> cells)</u>
CYANOKETONE (CYK; 25 $\mu$ M)	431 $\pm$ 35
CYK + P4 (0.1 $\mu$ M)	758 $\pm$ 26
CYK + P4 + A23187 (1.0 $\mu$ g/ml)	753 $\pm$ 23
CYK + P4 + FSH (75 ng/ml)	1928 $\pm$ 86

TABLE 9

Lack of effect of various concentrations of A23187 on conversion of exogenous progesterone (P4; 0.1 uM) to 20 $\alpha$ -OH-P (in the presence of cyanoketone (25 uM)) in granulosa cells during a 24 hour culture period. Values are means  $\pm$  SEM (n=8; two experiments).

<u>TREATMENT</u>	<u>20<math>\alpha</math>-OH-P (pg/10<sup>5</sup> cells)</u>
1. NONE	775 $\pm$ 85
2. A23187 (0.25 ug/ml)	987 $\pm$ 78
3. A23187 (0.50 ug/ml)	713 $\pm$ 124
4. A23187 (1.0 ug/ml)	394 $\pm$ 41
5. A23187 (2.0 ug/ml)	195 $\pm$ 12

Table 10

Influence of (Bu)<sub>2</sub>cAMP and cholera toxin on the conversion of exogenous progesterone (P<sub>4</sub>; 0.1 uM) to 20 $\alpha$ -OH-P (in the presence of cyanoketone (25 uM)) in granulosa cell 24 hour cultures. Values are the means  $\pm$  SEM (n=8; two experiments).

<u>TREATMENT</u>	<u>20<math>\alpha</math>-OH-P</u>
NONE	275 $\pm$ 38
(Bu) <sub>2</sub> cAMP (0.1 mM)	752 $\pm$ 76
Cholera Toxin (10 ng/ml)	1296 $\pm$ 178

TABLE 11

Effect of aminoglutethimide phosphate (AGP; 0.75 mM) on 20 $\alpha$ -HSD: Suppression of (Bu)<sub>2</sub>cAMP-, Cholera Toxin, and FSH-stimulated conversion of exogenous progesterone (P<sub>4</sub>; 0.1 uM) to 20 $\alpha$ -OH-P. Values are means  $\pm$  SEM (n=8; two experiments).

<u>TREATMENT</u>	<u>20<math>\alpha</math>-OH-P (pg/10<sup>5</sup> cells)</u>
A. NONE	228 $\pm$ 26
AGP	8.0 $\pm$ 1.2
AGP + P <sub>4</sub>	271 $\pm$ 15
AGP + P <sub>4</sub> + (Bu) <sub>2</sub> cAMP (0.1 mM)	179 $\pm$ 43
AGP + P <sub>4</sub> + Cholera Toxin (10 ng/ml)	216 $\pm$ 32
B. AGP + P <sub>4</sub>	379 $\pm$ 19
AGP + P <sub>4</sub> + FSH (75 ng/ml)	424 $\pm$ 24

12. Possible Requirement for Protein Synthesis in the Regulation of Progestin Production.

To study if the stimulation of granulosa cell progesterone production by FSH, A23187 and  $(\text{Bu})_2\text{cAMP}$  requires the synthesis of new proteins, initial experiments were performed to determine the highest concentrations of cycloheximide which would not affect basal progestin production. Basal progesterone production was not significantly attenuated by any of the concentrations of cycloheximide examined (28 ng/ml - 28 ug/ml;  $p > 0.05$ ). However, the two highest concentrations (2.8 and 28.0 ug/ml) did inhibit basal  $20\alpha\text{-OH-P}$  production ( $p < 0.001$ ). Therefore the highest concentration of cycloheximide employed in subsequent experiments was 280 ng/ml (Table 12).  $(\text{Bu})_2\text{cAMP}$  (0.1 mM) significantly stimulated both progesterone and  $20\alpha\text{-OH-P}$  in 24 hours ( $p < 0.0005$ ) (Table 13). Cycloheximide, in concentrations as high as 112 ng/ml, failed to attenuate the  $(\text{Bu})_2\text{cAMP}$ -stimulated progesterone or  $20\alpha\text{-OH-P}$  production in vitro ( $p > 0.05$ ). This agent however inhibited, in a concentration-dependent manner, the production of these steroids elicited by FSH and A23187 ( $p < 0.05$ ) (Fig. 10). Progesterone production appeared maximally inhibited at a cycloheximide concentration of 28 ng/ml, whereas inhibition of  $20\alpha\text{-OH-P}$  production was greater at 280 ng/ml of the inhibitor.

TABLE 12

Effect of different concentrations of cycloheximide (cyclohex) on basal progesterone (P4) and 20 $\alpha$ -OH-P production (picograms per 10<sup>5</sup> cells  $\pm$  SEM; n=8; two experiments) by granulosa cells cultured for 24 hours.

<u>TREATMENT</u>	<u>P4</u>	<u>20<math>\alpha</math>-OH-P</u>
NONE	157 $\pm$ 27.5	220 $\pm$ 13.0
CYCLOHEX (28 ng/ml)	117 $\pm$ 27.4	293 $\pm$ 10.8
CYCLOHEX (280 ng/ml)	311 $\pm$ 60.4	205 $\pm$ 5.5
CYCLOHEX (2.8 ug/ml)	140 $\pm$ 33.4	18.4 $\pm$ 1.7
CYCLOHEX (28.0 ug/ml)	158 $\pm$ 22.2	15.6 $\pm$ 1.4

TABLE 13

Lack of effect of cycloheximide (cyclohex) on (Bu)<sub>2</sub>cAMP-stimulated progesterone (P<sub>4</sub>) and 20 $\alpha$ -OH-P production in granulosa cells cultured for 24 hours. Values are means  $\pm$  SEM (n=8; two experiments).

TREATMENT	STEROID (pg/10 <sup>5</sup> cells)	
	P <sub>4</sub>	20 $\alpha$ -OH-P
1. NONE	76.4 $\pm$ 8.7	78.5 $\pm$ 2.7
2. (Bu) <sub>2</sub> cAMP (0.1 mM)	594 $\pm$ 37	1245 $\pm$ 125
3. (Bu) <sub>2</sub> cAMP + CYCLOHEX (28 ng/ml)	682 $\pm$ 86	994 $\pm$ 191
4. (Bu) <sub>2</sub> cAMP + CYCLOHEX (56 ng/ml)	663 $\pm$ 95	844 $\pm$ 100
5. (Bu) <sub>2</sub> cAMP + CYCLOHEX (112 ng/ml)	577 $\pm$ 75	872 $\pm$ 103

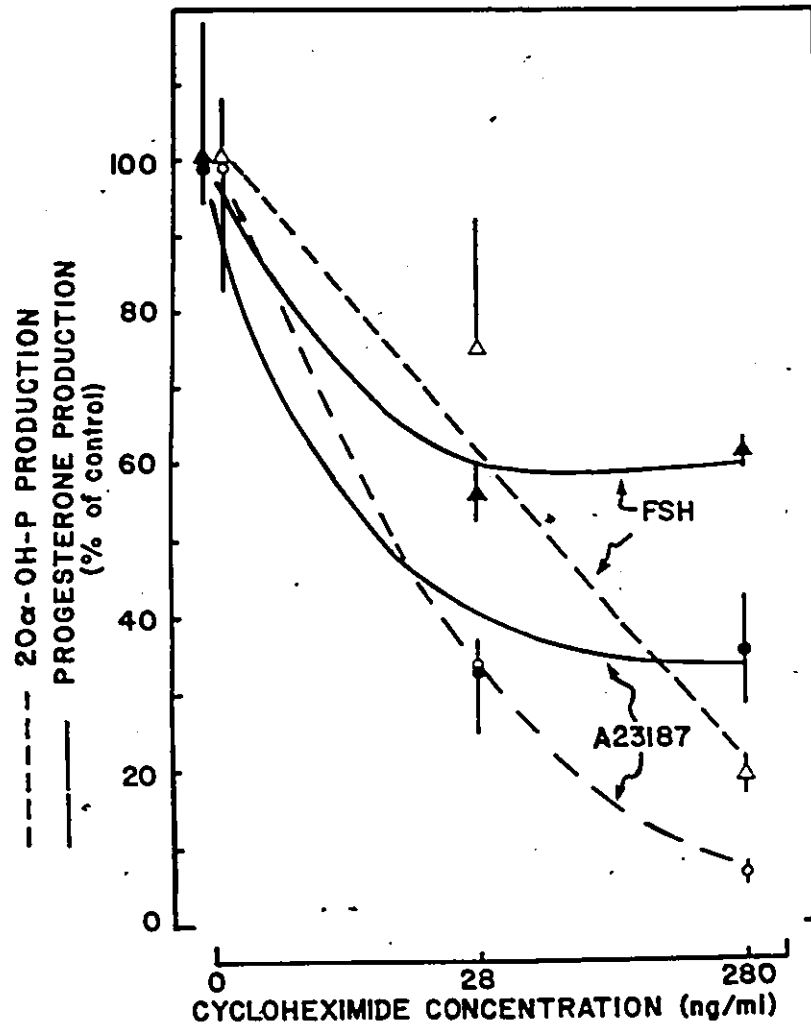


Figure 10.

Effects of cycloheximide on FSH - and A23187 - stimulated progesterone and 20α-OH-P production during 24h culture. Values are the means ± SEM (n=8; two experiments).

## Discussion

### (I) The Regulation of Progestin Production in Rat Granulosa

#### Cells in vitro: Possible Involvement of Calcium and Cyclic AMP.

Studies into the mechanism of regulation of granulosa cell progestin production have suggested an involvement of both cyclic AMP and calcium as mediators of gonadotropin actions.

Earlier studies by Tsang and Carnegie (1983) have demonstrated FSH-stimulated rise of cyclic AMP in the first two hours of granulosa cell tissue culture coinciding with increased progesterone production. Examination of the role of FSH in progestin production in the present studies revealed that this gonadotropin significantly stimulated both pregnenolone and progesterone production as well as synthesis of  $20\alpha$ -OH-P, the major metabolic breakdown product of progesterone. The stimulation of progesterone production by FSH was concentration- and time - dependent, with significant increase in the synthesis of the steroid noted six hours (earliest time examined) following exposure of the cells to the gonadotropin in vitro. Moreover, the effect of FSH on progesterone and  $20\alpha$ -OH-P production could readily be mimicked with the addition of  $(\text{Bu})_2\text{cAMP}$  to the culture medium. These findings, however, are in conflict with those of Trzeciak et al (1986) who failed to

detect FSH (50 ng/ml) - or (Bu)<sub>2</sub>cAMP (1.0 mM) - stimulated rises in progesterone or 20 $\alpha$ -OH-P levels until sometime between 24 and 48 hours of tissue culture. It is unclear why these results differ. Whether the apparent differences are due to the different stages of differentiation at which the granulosa cells were studied remains to be determined. In light of the present observation that (Bu)<sub>2</sub>cAMP increases the level of pregnenolone in 24 hour cultures, this action of FSH appears to involve a cyclic AMP mediated event. These findings are consistent with the observations of Trzeciak et al. (1986) that both FSH and (Bu)<sub>2</sub>cAMP stimulate the synthesis of the side chain cleavage enzymes with resulting increases in progestin biosynthesis (ie. pregnenolone, progesterone and 20 $\alpha$ -OH-P).

Previous studies in this laboratory (Carnegie and Tsang, 1983), and those of Veldhuis and Klase (1982), have demonstrated an involvement of Ca<sup>++</sup> in gonadotropic regulation of progestin production at a step distal to the cyclic AMP cascade. Results from the present studies show that the calcium ionophore A23187 can stimulate progesterone and 20 $\alpha$ -OH-P production without a significant increase in the synthesis of pregnenolone. These studies have provided the first evidence to suggest that the gonadotropic stimulation of pregnenolone synthesis may be calcium-independent.

The lack of a stimulatory effect of A23187 on pregnenolone production did not appear to be due to its rapid metabolism to progesterone as net accumulation of pregnenolone in granulosa cells was not affected by A23187 in the presence of cyanoketone. This is in contrast to a substantial FSH-stimulated pregnenolone production under identical experimental conditions. The possibility that A23187 could stimulate the conversion of pregnenolone to its metabolite  $17\alpha$ -OH-pregnenolone through an increased activity of  $17\alpha$ -hydroxylase was examined. Since antisera to  $17\alpha$ -OH-pregnenolone was unavailable, conversion of endogenous progesterone to  $17\alpha$ -OH-progesterone, also catalyzed by this enzyme, was measured. The inability of either A23187 or FSH to stimulate this conversion did not support this possibility. These findings are consistent with reports that  $17\alpha$ -hydroxylase is either absent or present at only low activity in granulosa cells (Bjersing and Cartensen, 1967; Taheri et al., 1986) and is not responsive to gonadotropins (Fevold 1983). Since A23187 failed to stimulate de novo pregnenolone production in the absence of cyanoketone, its stimulation of de novo progesterone and  $20\alpha$ -OH-P production suggests that there is sufficient pregnenolone substrate within the cells to support the increases in the steroidogenic responses. Veldhuis et al. (1984) concluded that  $Ca^{++}$  is important in LH-stimulated pregnenolone synthesis from endogenous sterol substrate in porcine granulosa cells, while cholesterol side-chain cleavage

was not significantly affected. Whether the apparent differences between these two observations and those of Veldhuis represent species differences and/or those of experimental design remains unclear.

Thus, while measurement of pregnenolone in the present studies demonstrated a lack of requirement for  $Ca^{++}$  in the synthesis of this steroid, the possibility that an increase in intracellular  $Ca^{++}$  may enhance substrate availability by rendering preformed but sequestered mitochondrial pregnenolone more accessible to 3BHS in the smooth endoplasmic reticulum cannot be excluded. It is proposed that calcium may regulate granulosa cell progesterone production in at least two ways:

- a) increasing activity of steroidogenic enzyme, ie. 3BHS, and
- b) enhancing substrate availability, ie. mitochondrial pregnenolone. Results from the present studies on progesterone production by granulosa cells from preantral and proestrus follicles (Figure 2 & 3) are consistent with this notion.

Recent work by Carnegie and Tsang (1987) indicates that treatment of granulosa cells with A23187 results in alterations of intracellular microtubule integrity and distribution, involving morphological changes in cell shape. The cells which

took on a typical flat and peripherally interdigitated appearance became more spherical with a smooth outline and occupying less area on the growth surface. It was postulated that this shape change might have altered distances between intracellular organelles and inclusions and hence might have optimized substrate availability. Carnegie and Tsang. (1987) have demonstrated similar findings in granulosa cells treated with FSH. The presence of tubulin binding sites on mitochondrial and plasma membranes and crossbridges linking microtubules and mitochondria has been observed (Bernier-Valentin, 1983, Smith et al. 1977; Heggeness et al., 1978). Soto et al. (1986) reported that human granulosa cells treated with 8-bromo-cyclic AMP and colchicine underwent changes in cell shape which were accompanied by the migration of mitochondria to a perinuclear location, apparently due to dismantling and reorganization of microtubules. However, a possible role of  $Ca^{++}$  in this regulation was not examined.

Closer examination of the role of  $Ca^{++}$  in A23187 stimulation of progesterone production involved depletion of extracellular  $Ca^{++}$  through chelation with EGTA. Previous work in this laboratory has demonstrated that incubation of rat granulosa cells with EGTA markedly reduced FSH - and cholera toxin- stimulated progesterone production without significant alteration in the net synthesis of cyclic AMP (Carnegie and Tsang, 1983).

Likewise, Janszen et al. (1976b) demonstrated that incubation of Leydig cell preparations with EGTA also suppressed LH-stimulated testosterone biosynthesis which was unaccompanied by a decline in protein kinase activity. Therefore, by rendering extracellular  $\text{Ca}^{++}$  inaccessible to appropriate subcellular locales and/or depleting intracellular  $\text{Ca}^{++}$  pools, EGTA is capable of severely reducing steroid production by directly influencing the activities of the steroidogenic enzyme(s) without affecting the cyclic AMP-protein kinase system. The concentration-dependent inhibition of A23187-stimulated progesterone production by EGTA attests to a  $\text{Ca}^{++}$ -specific action of the ionophore. However, the reason for biphasic nature of the response is unclear.

It is possible that the concentration of  $\text{Ca}^{++}$  in the culture medium (1.8mM) was supraoptimal such that addition of A23187 over-stimulated the influx of  $\text{Ca}^{++}$  and elicited a suboptimal response. The slight increase in the A23187-induced progesterone production noted at relatively low concentrations of EGTA is consistent with this possibility. The presence of  $\text{Ca}^{++}$ -dependent phosphodiesterases (PDE) in many tissues (Rasmussen and Goodman, 1977; Borle, 1981; Wolff and Brostrom, 1979) may also explain the stimulatory effect of EGTA as a consequence of PDE suppression.

(II) Calcium - Specific Regulation of 3BHSD in Granulosa Cells.

Zeleznik et al. (1974, 1979) have shown that FSH treatment of immature hypophysectomized female rats increases the activity of 3BHSD in ovarian granulosa cells. Dorrington and Armstrong (1979) and Jones and Hseuh (1981) have also demonstrated that treatment with FSH in vitro increases the conversion of labelled pregnenolone to progesterone in cultured granulosa cells. Results from the current studies are consistent with the aforementioned findings.

While our dose-response studies confirm the FSH stimulation of progesterone production from exogenous pregnenolone, our results extend these observations to provide direct evidence for the involvement of  $Ca^{++}$  in the regulation of the activity of this enzyme. Addition of the  $Ca^{++}$  ionophore, A23187, to rat granulosa cell cultures elicited a time - and concentration - dependent stimulation of the activity of 3BHSD, as evidenced by an increase in the conversion of exogenous pregnenolone to progesterone. The increase in net progesterone production from the exogenous precursor did not appear to be the result of decreased conversion of progesterone to its principle metabolite,  $20\alpha$ -OH-P, as production of the latter was likewise enhanced by the presence of the ionophore.

Results from the present investigations have shown that whereas FSH elicited a marked stimulation in progesterone production within six hours of incubation (earliest time examined) a significant increase in the ability of the cells to convert exogenous pregnenolone to progesterone could not be observed until after twelve hours of exposure to the gonadotropin in vitro, suggesting that a mechanism involving a time delay is operative in the gonadotropic regulation of 3BHS in granulosa cells. Furthermore, the time course of progesterone production in response to A23187 resembled that of the ionophore-induced increase in 3BHS activity and as observed with FSH stimulation, indicates a minimal requirement of 12 to 24 hours for these steroidogenic responses to be fully expressed. Since  $(\text{Bu})_2\text{cAMP}$  is capable of mimicking the action of FSH in granulosa cell pregnenolone production but not on the conversion of the latter steroid to progesterone, and that the reverse was true with regards to the action of A23187, the present studies suggest the possibility of two distinct but interdependent roles for cyclic AMP and calcium in the stimulation of progesterone synthesis by FSH in vitro: (a) a rapid, cyclic AMP - mediated response in pregnenolone production, and (b) a delayed calcium-dependent increase in 3BHS activity, resulting in an enhancement in the metabolism of pregnenolone to progesterone.

(III) Gonadotropic Regulation of 20 $\alpha$ -HSD.

Selective inhibition of 3 $\beta$ HSD with cyanoketone provided a valuable tool in the examination of the hormonal control of 20 $\alpha$ -HSD. The inability of A23187 to stimulate the metabolism of exogenous progesterone to 20 $\alpha$ -OH-P suggests a Ca<sup>++</sup> - independent mode of action of FSH in the regulation of 20 $\alpha$ -HSD activity. Stimulation of 20 $\alpha$ -HSD by human chorionic gonadotropin, GnRH, and prostaglandins has been well documented (Eckstein and Nimrod, 1979; Jones and Hseuh, 1981). The ability of FSH, (Bu)<sub>2</sub>cAMP, and cholera toxin to stimulate the conversion of progesterone to 20 $\alpha$ -OH-P indicates that 20 $\alpha$ -HSD is under gonadotropic regulation, possibly via a cyclic AMP-mediated mechanism. The role of 20 $\alpha$ -OH-P in mammals in vivo is not yet clear. While 20 $\alpha$ -OH-P is thought to be a relatively inactive progestin metabolite, evidence suggesting various physiologic functions for the steroid are beginning to emerge. Gilles and Karavolas (1981a, 1981b) have demonstrated that 20 $\alpha$ -OH-P facilitates ovulation and modulates serum gonadotropin and hypothalamic LHRH levels in the rat. Hilliard et al. (1967) has demonstrated a positive feedback action of the 20 $\alpha$ -OH-P in sustaining high levels of LH discharge in the mated rabbit. Whether the regulation of 20 $\alpha$ -HSD, and thus of progesterone and/or 20 $\alpha$ -OH-P levels may indeed be important in

controlling ovulation, gonadotropin secretion, and the maintenance and/or termination of pregnancy remains to be fully elucidated.

Our present observation that FSH and  $(\text{Bu})_2\text{cAMP}$  stimulate the metabolism of progesterone to  $20\alpha\text{-OH-P}$  are contrary to the findings of Moon et al (1985) who reported that gonadotropin and cyclic AMP analogue increased the conversion of  $(^3\text{H})20\alpha\text{-OH-P}$  to  $(^3\text{H})$  progesterone.

These results are difficult to reconcile considering that the in vitro experimental protocols were quite similar and that the antisera used were from the same source. One difference however was that the granulosa cells used by Moon and coworkers were obtained from preantral follicles which are less mature and differentiated from the proestrus follicular granulosa cells employed in the current studies. It is possible that follicular maturation and thus granulosa cell differentiation involves a change in the responsiveness of  $20\alpha\text{-HSD}$  to gonadotropin and cyclic AMP.

The use of AGP and cyanoketone has been a useful approach in inhibiting endogenous production of steroids including pregnenolone and progesterone. While AGP was found to have no significant influence on the basal conversion of exogenous

progesterone to  $20\alpha$ -OH-P it effectively attenuated this steroidogenic step in the presence of FSH,  $(\text{Bu})_2\text{cAMP}$  and cholera toxin.

The observation of the "non-specific" inhibition of these steroidogenic responses is an important finding and suggests that AGP may have a modulatory effect on steroidogenic enzyme(s) other than those involving the cytochrome P450 system. Caution should thus be exercised with the use of this inhibitor in the investigation of various steroidogenic processes. It is of interest to note that since basal production of  $20\alpha$ -OH-P from exogenous progesterone was not inhibited in the presence of AGP it is unlikely that the conversion of exogenous pregnenolone to progesterone in response to FSH and A23187 (3BHSD experiments) was a reflection of decreased metabolism of progesterone to  $20\alpha$ -OH-P.

#### (IV) Requirement for Protein Synthesis in the FSH Regulation of Granulosa Cell Progestin Production.

The synthesis of new protein is the basis of normal cellular function. A number of proteins produced by granulosa cells have been well defined. These include gonadotropin receptors (Rani et al., 1981), plasminogen activator and regulatory subunits of cyclic AMP-dependent protein kinase. Knecht and co-workers

have demonstrated FSH-stimulated RNA and protein biosynthesis in cultured rat granulosa cells. Using electrophoretic techniques to examine the gonadotropic control of the synthesis and secretion of cellular proteins in rat granulosa cells, Knecht et al (1986) recently found that FSH can exert both stimulatory and inhibitory effects on the production of specific proteins in a concentration-dependent manner. Furthermore, a number of the proteins synthesized under FSH stimulation were also apparent when cyclic AMP levels were elevated. Thus it appears that while cyclic AMP may be important in the synthesis of some cellular proteins, the refractoriness of  $(\text{Bu})_2\text{cAMP}$ -stimulated progesterone and  $20\alpha\text{-OH-P}$  production to cycloheximide noted in the present studies suggests that such stimulation is independent of the synthesis of new protein.

Previous studies with testicular and adrenal tissues have demonstrated a requirement for calcium in the synthesis of protein necessary for steroid hormone production (Hall & Eik-Nes 1962, Farese 1971 a, b). Whereas dependence on protein synthesis in LH-stimulated steroid production has been demonstrated in the ovary (Strauss III et al. 1981, Robinson et al. 1975, Hermier et al. 1971, Arthur & Boyd 1974; Toaff et al. 1979), a role for calcium in this process has not been established. Results from the present investigations also show

a requirement for protein synthesis in the gonadotropic regulation of ovarian steroidogenesis. Addition of cycloheximide to granulosa cell cultures markedly inhibited progesterone and  $20\alpha$ -OH-P production elicited by FSH in vitro.

The question arises as to why progesterone and  $20\alpha$ -OH-P production were not maintained by the FSH-stimulated increases in cellular cyclic AMP levels. Perhaps a dual mechanism is operative in the FSH action. While FSH may stimulate progesterone biosynthesis via increased production of cyclic AMP, a significant effect of the gonadotropin action may be mediated by a separate second messenger system involving the synthesis of new protein. The present observation that low concentrations of cycloheximide were capable of attenuating the steroidogenic responses elicited by A23187 is consistent with our earlier suggestion of a pivotal role of calcium in this process (Part I, Discussion). Moreover, our finding that cycloheximide inhibited A23187 - induced progesterone and  $20\alpha$ -OH-P production to a greater extent than the FSH-stimulated response is in good agreement with this hypothesis.

This idea is also consistent with our observed delay of 12 hours in the A23187-stimulated progesterone production and the conversion of exogenous pregnenolone to progesterone induced by either FSH or the ionophore. It is imperative however to note

that results generated by the use of cycloheximide are only suggestive of a requirement of protein synthesis, as cycloheximide may have non-specific inhibitory actions. Nevertheless, whether the protein(s) synthesized is 3BHSD or a modulator thereof is unclear. Further investigation involving the isolation and quantitation of the specific proteins following stimulation with FSH, cyclic AMP and/or calcium will undoubtedly provide a better understanding of the interactions of the second messenger systems in the gonadotopic regulation of steroid production in the rat granulosa cells.

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