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INFLUENCE OF PROTEIN KINASE C ACTIVATORS AND INHIBITORS ON RAT GRANULOSA CELL STEROIDOGENESIS IN VITRO

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Thesis submitted to the School of Graduate Studies of the University of Ottawa as partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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ABSTRACT

The present studies were undertaken to determine the involvement of protein kinase C (PKC) in the regulation of rat granulosa cell steroidogenesis in vitro. The effects of PKC activators [1-oleoyl-2-acetylglycerol (OAG); 1,2-dioctanoylglycerol (DiC_8) and phorbol 12-myristate 13-acetate (TPA)] and inhibitors [DL-Sphingosine (ESP) and 1-(5-Isoquinolinylsulfonyl)-3-methylpiperazine free base (H_7)] on basal and FSH-, $(Bu)_2$cAMP-, forskolin- and calcium ionophore A23187-stimulated pregnenolone (P_5), progesterone (P) and 20α-hydroxy-pregn-4-en-3-one (20α-OH-P) secretion by granulosa cells were studied in the absence or presence of inhibitors of steroidogenic enzymes (to separate the steps on the steroidogenic pathway).

Granulosa cells from immature Sprague-Dawley rats pretreated with pregnant mares' serum gonadotropin (PMSG) were preincubated for up to 20 h in the presence or absence of TPA (40 ng/ml) or OAG (20 μg/ml) and then incubated for up to 24 h with OAG (5-80 μg/ml), DiC_8 (5-80 μg/ml) or TPA (5-80 ng/ml) alone or in combination, in the presence or absence of FSH (9.4-300 ng/ml), $(Bu)_2$cAMP (1.5 mM), forskolin (1-100μM) or A23187 (0.25-1 μg/ml).

OAG, when continually present in the culture medium (MEM), significantly stimulated P_5, P and 20α-OH-P secretion during 6 to 24 h culture periods. It also markedly increased the conversion of exogenous P_5 to P and 20α-OH-P and exogenous P to 20α-OH-P during 24 h cultures. Pretreatment of granulosa cells with TPA for 1 h or treatment for up to 6 h resulted in a significant increase in P_5, P and 20α-OH-P secretion. Except for 20α-OH-P production, which was stimulated by the phorbol ester during all culture periods studied, secretion of P_5 and P (in the presence or absence of exogenous hormones and the inhibitors of steroidogenic enzymes) were substantially inhibited by TPA during a 24 h incubation. However, when granulosa cells were incubated with both OAG (20 μg/ml) and TPA (40 ng/ml), progestin secretion was increased irrespective of

the duration of incubation. PKC inhibitors dose-dependently suppressed the stimulatory effects of OAG (20 µg/ml) and TPA (40 ng/ml) with complete inhibition noted at 100 µM of H_7 and 10 µM of ESP.

Diacylglycerols and TPA exerted divergent effects on FSH-, $(Bu)_2$cAMP- and forskolin-stimulated progestin secretion. FSH-stimulated accumulation of P_5 throughout the culture periods (1-24 h) was markedly increased by OAG (20 µg/ml) but inhibited by TPA (40 ng/ml). OAG (5-80 µg/ml) and DiC_8 (20 µg/ml) significantly enhanced FSH-induced progestin secretion during 6 h and 24 h culture periods and increased steroid synthesis in 24 h cultures in the presence of $(Bu)_2$cAMP or forskolin. In contrast, TPA significantly inhibited FSH- and $(Bu)_2$cAMP- stimulated progestin secretion during both 6 h and 24 h of incubation. The influence of TPA (40 ng/ml) on forskolin- induced progestin secretion was biphasic and depended on the magnitude of stimulation by forskolin [TPA enhanced steroid secretion induced by low- (1-10 µM) but inhibited that induced by high- (50-100 µM) concentrations of forskolin].

The inhibitory action of TPA on FSH-stimulated progestin secretion was evident after 1 h of pretreatment with the phorbol ester, although the stimulatory effect of OAG was observed only during continual presence of this phospholipid. Pretreatment of granulosa cells with TPA (40 ng/ml) for 20 h to down-regulate PKC, decreased progestin secretion during subsequent incubation with FSH (150 ng/ml) and prevented any stimulation by OAG (20 µg/ml). The effects of OAG (20 µg/ml) and TPA (40 ng/ml) on FSH-induced steroid secretion appeared to be additive when both PKC activators were present together and differed significantly from those when OAG and TPA were present with FSH separately. In addition, OAG and TPA had also different actions on A23187-stimulated progestin secretion; while TPA (40 ng/ml) significantly reduced A23187-induced steroid secretion, OAG (20 µg/ml) was ineffective. Diolein (a nonpermeable diacylglycerol), 4α-phorbol 12,13-didecanoate and phorbol 13-monoacetate (two phorbol

esters with no tumor promoting activity) did not influence basal or FSH-stimulated steroid secretion by granulosa cells.

The present findings on the influences of PKC activators and inhibitors indicate a possible involvement of the enzyme in the regulation of ovarian steroidogenesis. Permeable diacylglycerols and TPA appear to have similar effects on basal steroid secretion during short (up to 6 h) culture periods, however, marked differences exist between the actions of these PKC activators on gonadotropin-, $(Bu)_2$cAMP-, forskolin- and A23187-stimulated granulosa cell steroid secretion in vitro. Possible reasons for the differential action of diacylglycerols and TPA might be their diverse effects on the PKC per se, the selective activation and/or down-regulation of various isoforms of the enzyme and/or dissimilar effects on intracellular Ca^{2+} concentration. In addition, exogenous diacylglycerols and TPA might nonspecifically stimulate signalling systems other then those of PKC and Ca^{2+}.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ACAT	acylCoA cholesterol acyltransferase
AGP	aminoglutethamide phosphate
ATP	adenosine triphosphate
$(Bu)_2cAMP$	N^6,O^2-dibutyryl cyclic 3',5'-adenosine monophosphate
cDNA	cloned deoxyribonucleic acid
CoA	coenzyme A
cAMP	cyclic 3',5'-adenosine monophosphate
DG	1-stearoyl-2-arachidonylglycerol
DHEA	dehydroepiandrosterone
DiC_8	1,2-dioctanoylglycerol
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid disodium salt
EGF	epidermal growth factor
ESP	DL-erythrosphingosine
FAD	flavin adenine dinucleotide
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
G	guanine
GAG	glycosaminoglycan
GnRH	gonadotropin-releasing hormone
GTP	guanosine triphosphate
H_7	1-(5-isoquinolinesulfonyl)-3-methylpiperazine
hCG	human chorionic gonadotropin
HDL	high density lipoprotein
hMG	human menopausal gonadotropin
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A-reductase
20α-HSD	20α-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
IL-I	interleukin-I

IP_3	inositol 1,4,5-trisphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
ISP	iron sulphur protein
LDL	low density lipoprotein
LH	luteinizing hormone
MEM	minimal essential medium
MIS	Mullerian inhibiting substance
mRNA	messenger ribonucleic acid
NADPH	dihydronicotinamide adenosine dinucleotide phosphate
OAG	1-oleoyl-2-acetylglycerol
20α-OH-P	20α-hydroxy-pregn-4-en-3-one
OMI	oocyte maturation inhibitor
P	progesterone (Δ^6-pregnene-3,20-dione)
P_5	pregnenolone
$P_{-450scc}$	cholesterol side-chain cleavage cytochrome P_{-450}
PA	phosphatidic acid
PBSG	phosphate buffered saline-gelatin solution
PDBu	phorbol dibutyrate
PDGF	platelet-derived growth factor
PGE	prostaglandin E
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin
PGS	prostaglandin synthase
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-monophosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipases A_2
PLC	phospholipase C
PLD	phospholipase D
PMSG	pregnant mares' serum gonadotropin
TGF	transforming growth factor
TNF	tumor necrosis factor
t-PA	tissue-type plasminogen activator
TPA	12-O-tetradecanoylphorbol 13-acetate
TSH	thyroid stimulating hormone

u-PA	urokinase-type plasminogen activator
VIP	vasoactive intestinal peptide

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INTRODUCTION

I. THE ANATOMY OF THE OVARY

A. Structure of the Ovary

The ovaries are nodular structures lying against the lateral wall of the pelvis and attached to the posterior surface of the broad ligament by a peritoneal fold called the mesovarium. The ovary consists of three structurally different regions: an outer cortex, a central medulla and an inner hilum around the point of attachment of the ovary to its mesentery (Goldfien and Scott, 1986; Ross, 1986).

The surface of the mature ovary is covered by a layer of germinal epithelium with the dense layer of connective tissue (tunica albuginea) below it. The cortex contains follicular structures at various stages of development (Fig. 1) and the connective tissue. The medulla consists of connective tissue stroma containing elastic fibres, blood vessels, nerves, lymphatics and smooth muscle fibres. The hilar region of the ovary contains clusters of large, steroid producing cells, resembling the Leydig or interstitial cells of the testes. Nerves, blood vessels and lymphatics traversing mesovarium penetrate the ovary at its hilum (Goldfien and Scott, 1986; Ross, 1986).

B. Ovarian Blood Supply

The ovary receives blood supply from branches of the ovarian and uterine arteries. These arteries enter the ovary through the mesovarium and hilum and divide into branches delivering blood to the stroma of the medulla and the cortex. Small arteries of the ovaries are characteristically spiral. The capillary blood gathers in a large, thin-walled plexus of

vessels (pampiniform plexus), then in an ovarian vein which leaves the ovary at its hilum. The plexus of arteries and capillaries (Goldfien and Scott, 1986) present in the theca interna of each developing follicle does not cross the basal lamina before ovulation (Ross, 1986).

The lymphatics first appear in the ovarian cortex, then spread to the medulla and leave the ovary at the hilum. Lymphatic channels abundant in theca externa, corpora lutea and corpora albicantia are absent in the theca interna, granulosa layer and tunica albuginea (Goldfien and Scott, 1986).

C. Ovarian Nerve Supply

The ovaries are innervated by the autonomic nervous system. The perikarya of preganglionic ovarian nerves arise form the lower thoracic segment of the spinal cord. The postganglionic fibers arise from the ovarian ganglion located at the origin of the ovarian artery or from the cell bodies of the celiac or renal plexuses. The rat ovary is innervated by the ovarian plexus nerve, which runs parallel to the ovarian artery and beside the large, superior ovarian nerve (SON). The latter runs in the suspensory ligament (Lawrence and Burden, 1980).

Both noradrenergic and cholinergic fibers have been found in the ovary (Lawrence and Burden, 1980; Jakobowitz and Wallach, 1987). Nerve fibers releasing other neurotransmitters, namely vasoactive intestinal peptide (VIP) (Larsson et al., 1977), somatostatin (McNeil and Burden, 1986), cholecystokinin-8 (McNeil and Burden, 1986), and neuropeptide Y (McDonald et al., 1987) have also been described in the rat ovary. The nerves supply the outer theca layer of developing follicle, but do not reach the granulosa cells (Burden, 1972; Jakobowitz and Wallach, 1987).

The nervous system seems to have the potential for direct modulation of follicular steroid biosynthesis. Ovarian nerves have been shown to have an inhibitory effect on

androgen production in the follicle (Morley et al., 1989). Through their effect on androgen synthesis, ovarian nerves might influence follicular development and atresia.

II. THE FUNCTION OF THE OVARY

The main functions of the ovary are oogenesis and the production of hormones necessary for sexual development, sexual cyclicity, implantation and the maintenance of pregnancy.

A. Structural and Morphological Changes in the Ovary During the Estrous Cycle

1) Folliculogenesis

The germ cells begin to proliferate during fetal life. At birth, the ovary is a nest of dictyate oocytes organized within primordial follicles (Richards et al.,1987). These follicles, consisting of a single layer of squamous epithelial cells surrounding the egg, represent a pool of nongrowing follicles (Fig. 1). The physiologic signal(s) involved in the selection and recruitment of particular primordial follicles into the developing pool is currently unknown. Selected follicles are transformed into primary follicles (Fig. 1), with the oocyte surrounded by a single layer of low columnar epithelium. Cells of the epithelium enter a proliferative phase and undergo mitotic divisions, leading to the formation of secondary follicles (Fig. 1). The stratified cuboidal epithelial cells of the secondary follicles are clearly recognized as granulosa cells. Centrally positioned and covered by the zona pellucida, the fully-grown (at the end of the secondary follicle stage) oocyte is surrounded by the layers of granulosa cells which communicate with each other and with the oocyte via gap junctions developed during proliferative stage of

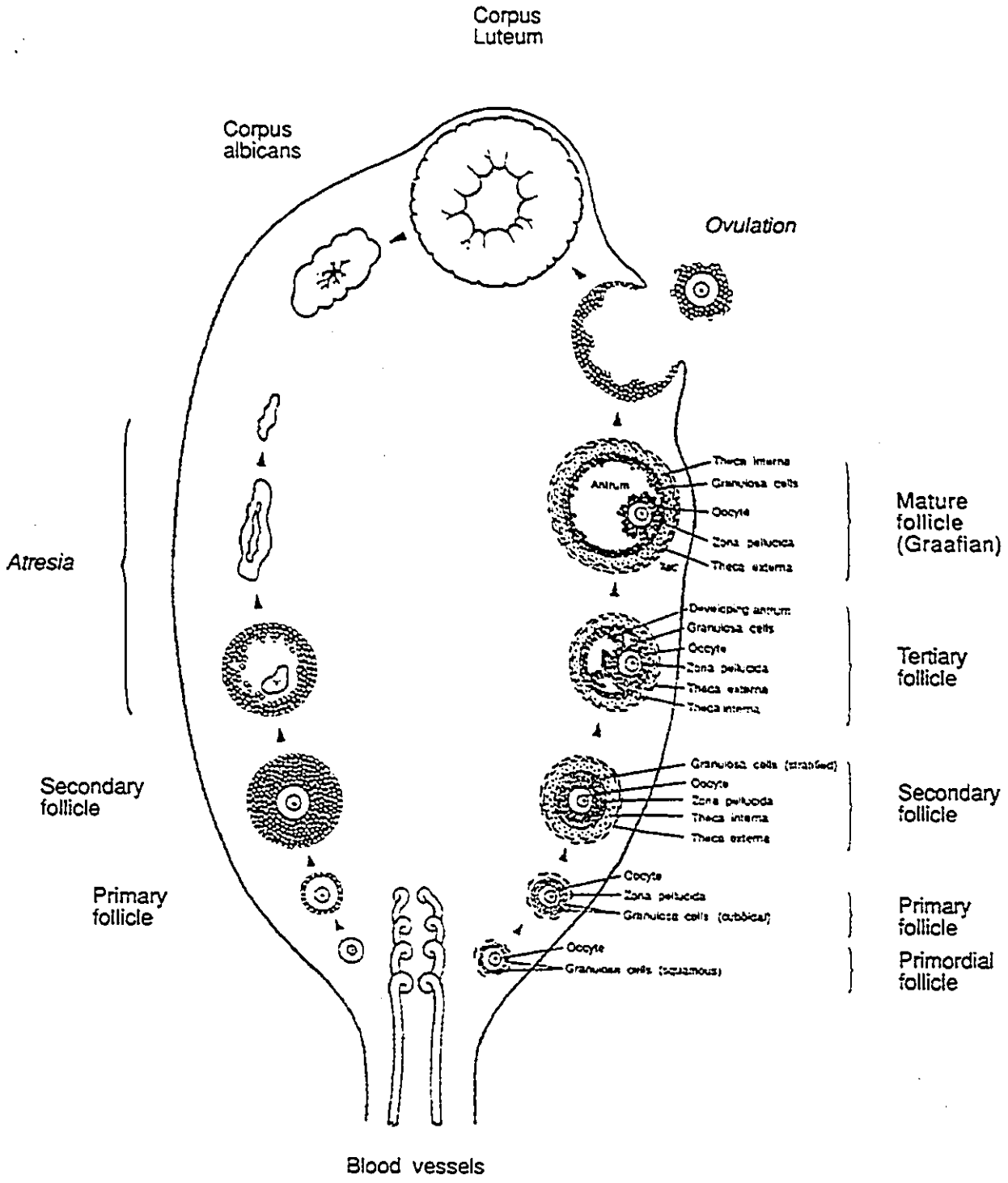

Figure 1 Diagram of developmental mammalian ovary. [Modified after Bruck-Kan (1979) and Meadows (1980)].

folliculogenesis (Burghardt and Matheson, 1982). Prior to ovulation, the granulosa cell layer transports nutrients by diffusion (Peters and McNatty, 1980). Transformation of primary follicles to secondary ones is accompanied by the development of high affinity receptors for follicle-stimulating hormone (FSH). The number of these receptors remains constant throughout follicle development (Presl et al.,1974).

Coincident with the proliferation of granulosa cells, migratory stromal cells condense on the outer side of the basal lamina and align themselves parallel to one another to form a ring around the follicle. These mesenchymal, spindle-like cells, which are initially indistinguishable from fibroblast cells (Ross, 1986), eventually form two distinct layers of theca cells. The portion of the theca adjacent to the basal lamina becomes the vascularized theca interna, while the fibrous portion merges with surrounding stroma - theca externa (Fig. 1) (Erickson, 1986; Freeman, 1988). Vascularization of the theca adjacent to basal lamina as well as the appearance of lymphatic spaces begin during growth of secondary follicles (O'Shea et al., 1980). The fibroblast-like cells acquire luteinizing hormone (LH) receptors (Midgley, 1973) and the 3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$isomerase enzyme complex (Magoffin and Erickson, 1982).

The multilayer secondary follicles develop into vesicular tertiary follicles (Fig. 1) characterized by the presence of multiple, fluid-filled intracellular spaces, which coalesce eventually to form the follicular antrum. The tertiary follicles increase markedly in size as a consequence of cell proliferation and fluid accumulation. The oocytes now surrounded by the cumulus granulosa cells, move to the side and occupy polar, eccentric positions in the follicles.

The growth and differentiation of follicular cells is regulated by a variety of endocrine, paracrine and autocrine factors, of which FSH and estrogen are indispensable. FSH is required for the induction of LH receptors in granulosa cells of preovulatory follicles (Richards et,,al., 1976; Richards and Williams, 1976). It also induces the formation of prolactin and catecholamine receptors in granulosa cells during the antral

stage of development. Receptors for estradiol, progesterone and testosterone appear in late secondary and tertiary follicles, providing granulusa cells with the competence to respond to these hormones (Erickson, 1986; Amsterdam and Rotmensch, 1987; Freeman, 1988). Estrogen, produced under the influence of FSH, appears to exert an autocrine effect on granulosa cells and is obligatory for their growth and differentiation (Franchimont et al., 1988, Richards and Hedin, 1988). It facilitates FSH action and allows granulosa cells to respond to relatively low concentrations of this gonadotropin (McNatty et,al., 1975; Richards and Hedin, 1988). The permissive influence of estrogens on cell proliferation is inhibited by prolactin, progesterone and reduced, nonaromatizable androgens (Franchimont, 1988). Androgens produced by theca-interstitial cells have a dual paracrine effect on follicle development. They serve as precursors of estradiol synthesis but also induce follicular atresia. The intensity and duration of FSH action are modulated by a variety of factors in the follicular fluid (Erickson, 1986).

Granulosa cells receive stimulatory or inhibitory messages by virtue of the presence of gap junctions and can respond in a synchronized manner. However, there is a growing body of evidence that the granulosa cell compartment is a complex tissue with a heterogeneous population of steroidogenic cells, which respond differently to the same physiologic stimulus (Erickson, 1986). In the preovulatory follicle, granulosa cells surround the encapsulated oocyte form the corona radiata, while the cumulus granulosa cells make contact with the corona radiata and the membrana granulosa cells, which line the basal lamina. The location of granulosa cells within the follicle appears to be associated with specific stages of cell differentiation and the degree of responsiveness to FSH. For example, although all granulosa cells possess FSH receptors, only membrana granulosa can respond to this gonadotropin by increasing of aromatase (Zoller and Weiss, 1970) and LH and prolactin receptors (Zeleznik et al., 1974; Oxberry and Greenwald, 1982).

Coincident with the development of granulosa cells of tertiary (Graaffian) follicles, the theca cells also undergo differentiation. Under the influence of LH, some of the spindle-shaped cells nearest the basal lamina acquire increased amounts of cytoplasm and appear large, rounded and epithelioid. These cells, called theca-interstitial, (Eshkol et al., 1970) accumulate in the theca interna (Erickson et al., 1985) and become highly differentiated endocrine cells with the capacity to synthesize and secrete androgens (mainly androstenedione) (Bleil and Wasserman, 1980). Besides LH, locally produced prostaglandin E_2 (PGE_2) can also induce theca-interstitial cell differentiation and stimulate androgen production (Erickson et al. 1985). The stimulatory action of LH and PGE_2 can be modulated by other physiologic regulators (Erickson et al., 1985). Similarly to their role in granulosa layers, gap junctions are crucial for thecal cell-cell communication. The theca externa differentiate into smooth muscle cells (Erickson et al., 1985) which are innervated by sympathetic and parasympathetic nerves (Bahr and Kao, 1974). These muscle cells are believed to play a role in ovulation (Lipner, 1973; Espey, 1978).

2) Oogenesis

During fetal life the germinal cells develop and multiply, giving rise to the oogonia. At birth, the oogonia enter meiosis but stop a few days later at the diplotene stage of prophase (Erickson, 1986; Richards, 1987; Franchimont et al., 1988). During meiotic arrest, individual chromosomes disorganize, chromatin dissolves and becomes surrounded by a membrane to form the germinal vesicle (Franchimont et al., 1988). The oogonia remain in this quiescent stage until selection of the follicles for development. The mechanism and trigger for the selection are not fully understood. Reactivation of the oocyte genome starts at the beginning of the proliferative stage of follicular development and the transformation of the low columnar epithelium to cuboidal shape. At this stage, a substantial increase in oocyte size and morphological changes occur. These changes include the breakdown of the Balbian body, dispersion of mitochondria, replication of

Golgi apparatus and the formation of the zona pellucida (Erickson, 1986). The growth and morphological changes of the oocyte stop at the end of the secondary follicle stage (Freeman, 1988), while the follicle size continues to increase substantially. The final maturational step of oogenesis (first meiotic division and release of the first polar body) begins within the follicle and is induced by the preovulatory LH surge (Erickson, 1986; Franchimont et al., 1988). Meiosis stops at metaphase of the second meiotic division and resumes only after fertilization (Freeman, 1988; Franchimont et al., 1988).

The trigger for the resumption of the oocyte maturation is poorly understood. However, the presence of granulosa cells is obligatory for oocyte maturation. Corona radiata granulosa cells are coupled to the oocyte via gap junctions and are important sources of nutrients and various regulatory substances (Eppig, 1980; Bachranova, 1980). The latter include Mullerian Inhibiting Substance (MIS) (Tsafriri, 1978), and Oocyte Maturation Inhibitor (OMI) (Tsafriri et al., 1982) which regulate meiotic divisions of the oocyte in opposite ways. The luteinizing hormone surge is effective in inducing the resumption of the oocyte maturation, irrespective of the presence of OMI. The decrease in the OMI levels (Van de Wiel et al., 1983) and the increased synthesis of estrogens and progesterone during follicular maturation, together with the appearance of MIS (Takahashi et al., 1986), may also be important factors for the resumption of meiosis. The formation of OMI is stimulated by FSH (Anderson and Stone, 1980) and prolactin (Channing and Evans, 1982) but inhibited by LH and androgens (Franchimont et al., 1988). Mori et al. (1984, 1985) reported that somatostatin inhibits spontaneous meiosis and may play the role of OMI. Cyclic adenosine monophosphate (cAMP) analogs also inhibit spontaneous or FSH-induced meiosis (Miller and Behrman, 1987; Eppig and Downs, 1984). In addition, estrogen appears to be essential for normal oocyte maturation. High levels of this steroid facilitate complete differentiation of the egg (Erickson, 1986).

3) Atresia

Atresia is a degenerative process leading to the destruction of the follicle. It may occur at any stage of follicular development and is clearly visible during each cycle (Freeman, 1988; Erickson et al., 1985). It is characterized by pyknosis (shrinking of the nuclei and chromatin) of granulosa cells, rupture of the nucleus and disintegration of chromatin (Freeman, 1988), loss of gap junctions, loss of granulosa cell LH and FSH receptors and lipid accumulation in follicular cells (Byskov, 1979). The final result of atresia is death of the oocyte and granulosa cells. The theca interna of degenerating follicles however, remains viable in many species and gives rise to secondary interstitial cells. Groups of these cells are scattered within the medulla and respond to LH with androstenedione production (McNatty et al., 1979).

Atresia is believed to be controlled by several endocrine, paracrine and autocrine mechanisms. While FSH (Byskow, 1979) and estrogens (Richards, 1975; Hillier and Ross, 1979) prevent atresia, LH (Hillier and Ross, 1979; Terranova, 1981) and androgens induce follicular degeneration (Payne and Rusner, 1958; Hillier and Ross, 1979). According to Terranova (1981), LH induces atresia by causing a switch from the synthesis of androgen to progesterone in theca-interstitial cells, thus leading to decreased estrogen production. Testosterone induces atresia by promoting the loss of estrogen receptors and decreasing the sensitivity of granulosa cells to estrogen (Saidudolin and Zassenhaus, 1978). Prolactin, progesterone and FSH receptor binding inhibitor, follicular regulatory protein(s) and gonadal gonadotropin-releasing hormone (GnRH) inhibit aromatization and decrease estrogens content in granulosa cells (Franchimont et al., 1988). Since estrogen plays a pivotal role in follicle growth and development, the decrease in follicular content of this hormone prevents further development and facilitates atresia. The mechanism(s) involved in the initiation and control of atresia is (are) not known.

4) Ovulation

Ovulation is the rupture of the mature ovarian follicle leading to the release and expulsion of the egg. The precise mechanism involved in the regulation of ovulation is not fully understood. Nevertheless, it is known that thinning of the follicular wall caused by degradation of the connective tissue occurs in the apex region of the follicle. This event is linked to a reduction in collagen and proteoglycan synthesis and an increase in the activity of proteolytic enzymes (Yanagishita and Hascall, 1979; Reich et al., 1983). The level of collagen and proteoglycans synthesis decrease with the increase in follicle size. In addition, it is believed that contraction of the follicular smooth muscle plays a crucial role in the collapse of the follicle and the expulsion of the egg (Erickson, 1986).

The most important factor leading to ovulation is LH (Erickson, 1986; Franchimont et al., 1988). The preovulatory surge of LH increases ovarian PGE and $PGF_{2\alpha}$ content. An increase in prostaglandins appears to be an obligatory event in LH-induced ovulation in the rat, rabbit and pig (Ainsworth et al., 1975; Clark et al., 1978; Tsang et al., 1979; Espey, 1980; Holmes et al., 1986). LH, FSH and prostaglandins stimulate plasminogen activator (PA) synthesis and secretion by granulosa cells (Richards, 1988). The content and the activity of PA's rises dramatically (3-14 fold) prior to the rupture of the follicle. Plasminogen activator catalyses the breakdown of plasminogen to plasmin, which in turn activates the conversion of procollagenase to collagenase, an enzyme involved in the dissolution of the basal lamina and perifollicular stroma during ovulation (Canipari and Strickland, 1985; Wang and Leung, 1983). In addition to gonadotropins and prostaglandins, progesterone (Downs and Long, 1983), estradiol (Reich et al., 1986) and relaxin (Bryant-Greenwood, 1982) have also been shown to stimulate PA and collagenase activities in rat granulosa cells in vitro.

5) **Luteinization-Corpus Luteum Formation**

The corpus luteum contains granulosa and theca cells of the preovulatory follicle. Luteinization of these cells is triggered by the LH surge. Unlike ovulation, luteinization does not require the synthesis of prostaglandins (Richards, 1975). Luteinized granulosa cells stop proliferating, enlarge, gain lipids and synthesize increased amounts of progesterone, necessary for the maintenance of pregnancy. In the rat, each corpus luteum can be distinguished on the basis of size, vascularity, histological and staining characteristics (Freeman, 1988). The corpora lutea regress suddenly in diestrous of the second cycle, coinciding with the closure of blood vessels, degeneration of cells, leukocyte infiltration, increased content of 20α-hydroxysteroid dehydrogenase and increased cholesterol content (Freeman, 1988). These so called "nonfunctional" corpora lutea (present in unmated animals) produce relatively low levels of progesterone for 1-2 days. If the ovulated eggs are fertilized, the corpora lutea are maintained throughout pregnancy in the rat (20-22 days).

B. Steroidogenesis in the Ovary

The mammalian ovaries synthesize and secrete a variety of biologically active substances, of which the best known are steroid hormones. These hormones have marked effects on their target tissues in the reproductive, nervous, muscular, skeletal, cardiovascular and immune systems. They also have important effects on the liver, adipose and cutaneous cells. In addition to their regulatory role in sexual maturation and the development of secondary and tertiary sex characteristics, steroid hormones are important for pubertal growth and development.

The paracrine and autocrine actions of steroids are crucial for oocyte maturation. Steroids facilitate the passage of the oocyte-cumulus complex through the oviduct and the transport of spermatozoa to the site of fertilization. They prepare the endometrium for

implantation and embryonic development and, maintain pregnancy until delivery at term (Gore-Langton and Armstrong, 1988).

The rat ovarian follicles synthesize and secrete three classes of steroid hormones: progestins, androgens and estrogens . Granulosa cells secrete progesterone (P) and estradiol. Theca cells produce P and androgens, of which the latter serve as precursors for estrogen synthesis in granulosa cells.

The biosynthetic pathway of steroids and the subcellular localization of major steroidogenic enzymes is in general similar in most steroid producing cells such as those of the ovary (Fig. 2), testis and adrenal. All steroidogenic tissues utilize cholesterol as a substrate for the synthesis of pregnenolone (P_5). Ovarian theca interna cells possess the enzymes necessary for the production of androgens and, in some species (horse, pig and monkeys), also the enzymes involved in the synthesis of estrogens (aromatase). Rat granulosa cells are deficient in the enzymes responsible for the metabolism of progestins to androgens (17 α hydroxylase and $C_{17,20}$-lyase) but have the ability to aromatize androgens to estrogens (Gore-Langton and Armstrong, 1988; Hsueh et al., 1989). Luteal cells contain those enzymes required for the production of progestins and estrogens (Hsueh et al., 1989).

1) Steroidogenic Pathways

a. Cholesterol as a Common Substrate for Steroidogenesis

A common precursor of the synthesis of all steroid hormones is cholesterol. Steroidogenic tissues are capable of obtaining this lipid from three sources: 1) de novo synthesis from 2-carbon components derived from the cellular metabolism of carbohydrates, fats or proteins, 2) cholesterol esters, free cholesterol or cholesterol as a part of cellular membranes, 3) dietary cholesterol in the form of lipoproteins delivered to the cells by the circulation (Gore-Langton and Armstrong, 1988) (Fig. 2).

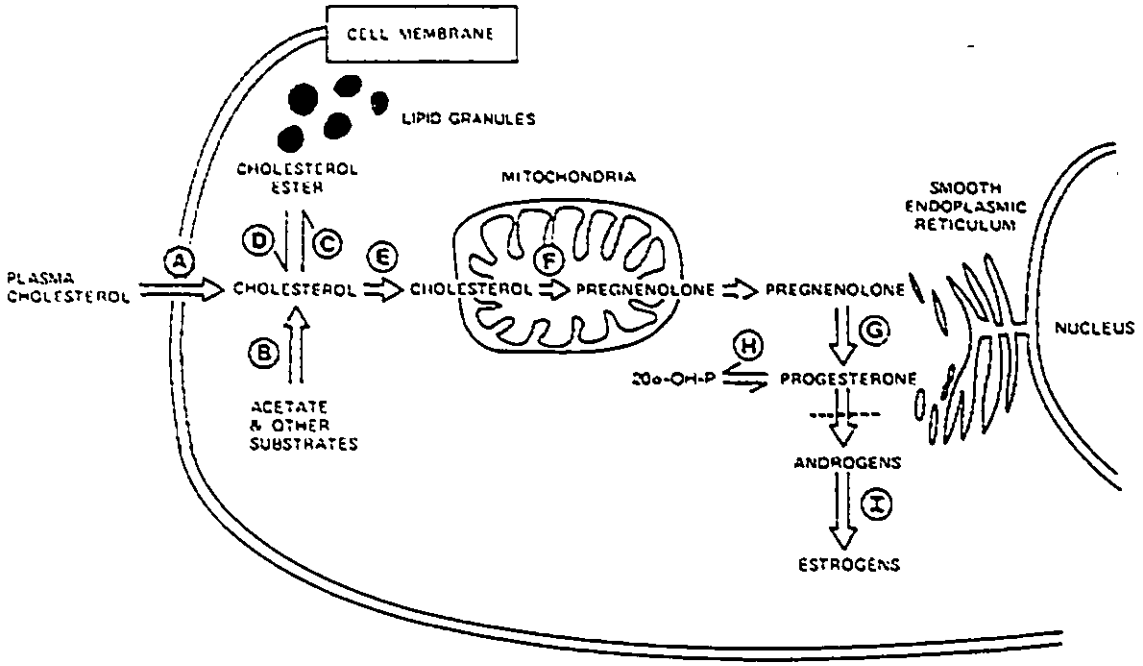

Figure 2 Pathway for biosynthesis of pregnenolone, progestins and estrogens in rat granulosa cells: diagrammatic representation of cellular organelles and key enzymes involved in steroid synthesis. [After Hsueh et al. (1983)]. Dashed line between progesterone and androgens emphasizes the deficiency of 17β-hydroxylase and 17,20-desmolase in granulosa cells.

A. lipoprotein receptors
B. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase)
C. acyl-coenzyme A: cholesterol acyl transferase (ACAT)
D. cholesterol esterase
E. cholesterol transport to the mitochondria
F. cholesterol side-chain cleavage enzymes (phospholipid membrane environment and enzyme levels)
G. 3β-hydroxysteroid dehydrogenase (3β-HSD)
H. 20α-hydroxysteroid dehyrogenase (20α-HSD)
I. aromatase

Although cholesterol from lipoproteins seems to be the main precursor for steroidogenesis, its availability and utilization differ between various cellular compartments of the ovary. The theca interna and the corpus luteum each have an abundant blood supply and thus easy access to lipoproteins. However, the granulosa cells, which are surrounded by a basement membrane impermeable to lipoproteins during follicular development, acquire their cholesterol via de novo synthesis of acetylcoenzyme A. After the preovulatory LH surge, the luteinized granulosa cells are supplied with lipoproteins through a network of blood vessels. Both low density lipoproteins (LDL) and high density lipoprotein (HDL) have been shown to be effective precursors for steroidogenesis (Gwynne and Strauss III, 1982). Human steroidogenic cells derive most of their cholesterol from LDL (Brown and Goldstein, 1986), although in rodents such as the rat, the primary source of cholesterol is HDL (Gwynne and Strauss, 1982). Upon reaching the cells, lipoproteins bind, via their apoprotein component, to specific cellular receptors localized in coated pits. The lipoprotein-receptor complex is internalized (by receptor-mediated endocytosis) in the form of coated vesicles which coalesce inside the cell and form endosomes. Due to a decrease in the endosomal pH mediated by the action of an adenosine triphosphate (ATP)-driven proton pump, the internalized complex dissociates to lipoprotein and the receptor. The latter clusters with other receptors in a segment of the endosomal membrane that pinches off to form a recycling vesicle. The receptors are then recycled to the cellular membrane and gathered in coated pits undergo subsequent endocytosis. The LDL is delivered to the lysosome where the protein component is hydrolized to amino-acids, and the cholesterol esters to free cholesterol (Brown and Goldstein, 1986). HDL cholesterol is taken up by a mechanism which may include endocytosis of HDL via LDL receptor, although a pathway for uptake of free and estrified cholesterol of HDL independent of LDL receptor has also been demonstrated (Veldhuis and Gwynne, 1989; Rajan and Menon, 1989).

Free cholesterol is metabolized to steroid hormones, or is esterified by acyl-coenzyme A (acyl-CoA) cholesterol acyltransferase (ACAT) and stored in lipid droplets within the cell (Fig. 2). The equilibrium between free and stored cholesterol is regulated by the balance between ACAT (cholesterol ester synthetase) and sterol ester hydrolase (cholesterol esterase). The activities of these enzymes and that of 3-hydroxy-3-methylglutaryl CoA (HMG CoA)-reductase, the rate limiting enzyme in de novo cholesterol biosynthesis, are regulated by cholesterol generated from lipoproteins. Cholesterol from LDL is believed to be responsible for suppressing HMG CoA reductase activity (Luskey et al., 1988). By lowering the concentration of receptor mRNA, cholesterol also suppresses synthesis of LDL receptors and prevents cholesterol over-accumulation (Brown and Goldstein, 1986).

The expression of LDL receptors, internalization and degradation of lipoproteins as well as activities of enzymes involved in cholesterol synthesis, esterification and deesterification are under hormonal control (Golos et al., 1987b; Golos, 1988). Various factors, including steroid hormones (Veldhuis and Gwynne, 1985), insulin (Veldhuis et al., 1987) and insulin-like growth factors (Veldhuis et al., 1987) modulate LDL metabolism in granulosa cells. Gonadotropins stimulate the activity of cholesterol ester hydrolase and inhibit ACAT (Miller, 1988), thus increasing the availability of free cholesterol for steroid hormone synthesis. Since free cholesterol is relatively insoluble in aqueous cytosol, it is transferred from lipid droplets to mitochondria (and from the outer to the inner mitochondrial membrane) by sterol carrier proteins of which the best known is sterol carrier protein 2 (SCP-2) (Vahouny, 1987; Steinschneider et al., 1989).

b. Cholesterol Side-Chain Cleavage: Formation of Pregnenolone

The synthesis of all steroid hormones begins with the cleavage of the $C_{20,22}$ bond of cholesterol to form the key steroidogenic intermediate, P_5 and the 6-carbon fragment, isocaproic acid. This first and rate limiting step of steroidogenesis is catalyzed by a

mitochondrial enzyme complex composed of three components: cholesterol side-chain cleavage cytochrome P-450 ($P_{-450scc}$), a flavin adenine dinucleotide (FAD)-containing flavoprotein and the sulfur-containing heme protein called ferredoxin (luteodoxin in luteal and adrenodoxin in adrenal cells) (Strauss et al., 1981) (Fig. 2). $P_{-450scc}$ functions as the terminal (electron-acceptor) oxygenase in a mitochondrial electron transport system. Electrons from nicotinamide adenosine dinucleotide phosphate, reduced form (NADPH) generated within the mitochondria by the Krebs cycle are accepted by ferredoxin reductase. This reductase transfers the electrons to ferredoxin, which shuttles them from flavoprotein to $P_{-450scc}$ (Farkash et al., 1986, Gore-Langton and Armstrong, 1988). According to Lieberman et al. (1984), transient hydroxylated intermediates of cholesterol side-chain cleavage process remain bound to $P_{-450scc}$ until P_5 is released. The content and the activity of $P_{-450scc}$ present in the mitochondria are greater in preovulatory than in the small, preantral follicles, but greatest in corpora lutea (Zlotkin et al., 1986). During luteinization,the content of cytochrome P_{-450} increases markedly, while ferredoxin levels remain only slightly elevated (Richards and Hedin, 1988).

c. Metabolism of Pregnenolone: Formation of Progesterone

P_5, produced during cholesterol side-chain cleavage, leaves the mitochondria and is translocated to the endoplasmic reticulum, for further metabolism. It is converted to P by a microsomal enzyme complex: Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD)/Δ^{5-4}isomerase (Fig. 2). Since the activities of these enzyme components could not be dissociated in mammalian steroidogenic tissues, they are considered to be a physiologic entity (Hall, 1984). Although indirect enzymatic studies suggested that there is a single 3β-HSD enzyme and two or three distinct isozymes of isomerase (Penning and Covey, 1982; Gower and Cooke, 1983), more recent data indicate the presence of a single protein mediating both 3β-HSD and isomerase activities in the adrenals and the testes (Ishi-Ohba et al., 1986). The Δ^5-3β-hydroxysteroid dehydrogenase/Δ^{5-4}isomerase utilize

NAD^+ as an electron acceptor. The reaction, involving a shift of a double bond from Δ^5 to Δ^4 position, is irreversible under physiologic conditions (Gore-Langton and Armstrong, 1988)

A similar but not identical enzyme complex is responsible for the metabolism of 17α-OH-pregnenolone to 17α-OH-P and dehydroepiandrosterone to androstenedione. In the rat conversion of P_5 to P is a rate limiting step and can be inhibited by cyanoketone(Hsueh et al., 1989).

d. Metabolism of progesterone: Formation of 20α-hydroxy-pregn-4-en-3-one

The metabolism of P in granulosa cells primarily involves the enzyme 20α-hydroxy steroid dehydrogenase (20α-HSD), which reversibly converts the hormone to the considerably less active 20α-hydroxy-pregn-4-en-3-one (20α-OH-P) (Fig. 2). Granulosa cells lack significant levels of 17α-hydroxylase and $C_{17,20}$-lyase required for the conversion of P to androgens. Since these enzymes are mainly found in the theca-interstitial cells, it is believed that P produced in granulosa cells diffuses across the basement membrane to the theca cell layer for synthesis of C_{19} androgens (Tsang et al., 1987). The androgens are shuttled back to the granulosa cells where they are metabolized to C_{18} estrogens by the NADPH-dependent aromatase enzyme complex (Armstrong and Dorrington, 1976; Tsang et al., 1985).

e. C_{21} Steroid Side-Chain Cleavage: Formation of Androgens

Both P_5 and P may be converted to C_{19} androgens. This reaction is catalyzed by a rate limiting enzyme complex located in the smooth endoplasmic reticulum. The enzyme complex is composed of the cytochrome $P_{-450_{17}}$ and the flavoprotein NADPH-cytochrome P_{-450} reductase. Although $P_{-450_{17}}$ has long been regarded to consist of activities of two enzymes, 17α–hydroxylase and $C_{17,20}$-lyase, recent studies have emphasized a functional but not genetic or structural distinction between them (Hsueh et

al., 1989). 17α–hydroxylation and cleavage of $C_{17,20}$ bond of P_5 leads to formation of dehydroepiandrosterone (DHEA) through the route referred to as the 5-ene-3β hydroxy (or Δ^5) pathway. DHEA is converted by 3β–HSD/isomerase to androstenedione. Androstendione is the most abundant androgen secreted by the ovary but is much less potent than testosterone. It can be converted to testosterone by 17β–hydroxysteroid dehydrogenase (Bjersing, 1967).

An alternate route of androgen production, which involves the 17α-hydroxylation and cleavage of $C\text{-}_{17,20}$ bond of P leads to the formation of androstenedione through the 4-en-3-oxo (Δ^4) pathway. However, it is not clear whether the enzymes of Δ^4 and Δ^5 pathways are identical (Gower, 1984). In addition, the ovary may produce C_{19} steroids without prior hydroxylation. Androst-5,16-dien-3β–ol and androst-4,16-dien-3-one which are formed during this reaction have been found in porcine and human follicular fluids (Gower, 1984).

f. Aromatization of C_{19} - Steroids: Formation of Estrogens

Estrone and estradiol are formed from androstenedione and testosterone. The reaction takes place in the agranular endoplasmic reticulum and is catalyzed by the enzyme complex referred to as aromatase (Fig. 2). The aromatase is composed of specific cytochrome P_{-450} enzyme (P_{-450} aromatase) and the NADPH-cytochrome C reductase. Aromatization is an irreversible, multiple step reaction. The aromatase converts androgens to estrogens by hydroxylation of the C_{19} methyl group and of the C_2, resulting in the loss of C_{19} and the aromatization of the steroid A ring (Fishman and Goto, 1981; Fishman and Rajo, 1981). This series of reactions requires 3 moles of 0_2 and 3 moles of NADPH per mole of estrogen formed. According to Lieberman et al. (1984), the intermediates of the reaction are bound to the enzymes throughout the aromatization process.

In mammals estradiol is the most potent of estrogens. Estrone can be converted to estradiol by the 17β–hydroxysteroid dehydrogenase.

g. 5α-Reduced Metabolites of Androgens and Progestins

The conversion of C_{21} and C_{19} steroids to 5α–reduced metabolites is catalyzed by the NADPH-dependent 5α–reductase associated with the nuclear membrane (Moore and Wilson, 1972). Although indirect studies imply the presence of two isozymes (Martini et al., 1986), molecular biological findings clearly indicate, at least in humans, the presence of a single gene for this enzyme (Wilson, 1987). 5α-reduction is followed directly by stereospecific reduction of the 3-oxo-group by the 3α–hydroxysteroid dehydrogenase and the formation of 3α–OH-derivatives of the 5α–reduced metabolites. 5α– and 3α–reductions may occur before or after cleavage of the $C\text{-}_{17,20}$ bond (Inaba et al., 1979). 3β-OH-derivatives of 5α–reduced progestins and androgens are present in ovarian homogenates and circulating levels of 5α–androstane-3β, 17β–diol are observed in the circulation of prepubertal rats (Gore-Langton and Armstrong, 1988).

2) Steroidogenesis in Granulosa Cells

a. Estrogen production

Biosynthesis of estradiol requires androgen precursors as the substrates for aromatization. With the exception of those from the bovine ovary, granulosa cells of all species studied do not have significant enzyme activities necessary for cleavage of C_{21} steroids and the production of C_{19} androgens (Gore-Langton and Armstrong, 1988). According to the "two cell, two gonadotropin" theory, androstenedione and testosterone synthesized by the theca-interstitial cells of preovulatory and antral follicles traverse the follicular basement membrane and are aromatized by microsomal aromatase in the granulosa cells. In vitro studies support this concept. Studies indicate that exogenous

aromatizable androgens are essential for estrogen production by granulosa cells in vitro (Dorrington et al., 1975; Moon et al., 1978). Coincubation of granulosa and theca cells has demonstrated their synergism in estrogen synthesis (Liu and Hsueh, 1986). While androstenedione is the predominant androgen synthesized by the theca-interstitial cells, estradiol is the major estrogen produced by the granulosa cells. 17β-hydroxysteroid dehydrogenase is responsible for the conversion of androstenedione to testosterone and of estrone to estradiol.

Studies indicate that 17-ketosteroid reductase is a constitutive enzyme present in granulosa cells irrespective of the stage of follicle development (Bogovich and Richards, 1984) and is more abundant in granulosa cells than in theca cells. This ensures that the conversion of androstenedione to testosterone takes place mainly in close proximity to the site of aromatization of testosterone to estradiol, thus minimizing circulating levels of androgens (Richards et al., 1987). In rat ovarian follicles, the aromatase is localized exclusively in granulosa cells (Gore-Langton and Armstrong, 1988). Rat P_{-450} aromatase mRNA is present at low levels in the granulosa cells of preovulatory follicles and is significantly increased in the corpora lutea of pregnant rats (Richards et al., 1987). The elevated mRNA content in corpora lutea corroborates the findings of high aromatase activity in rat corpus luteum in vivo. Thecal androgens which do not undergo aromatization due to the absence or low levels of the aromatase enzyme may be reduced by 5α-reductase and 3α-hydroxysteroid dehydrogenase present in the granulosa cells. Unlike other enzymes involved in the metabolism of steroid hormones in granulosa cells, 5α-reductase activity does not seem to be regulated by gonadotropins (Dorrington and Armstrong, 1979).

b. Production of progestins

Granulosa cells produce P and its metabolite 20α-OH-P either from cholesterol synthesized "de novo" or from lipoprotein-associated cholesterol (Veldhuis et al., 1984;

Baranao and Hammond, 1986; Grummer and Carrol, 1988). Granulosa cells have the enzymes required for cholesterol synthesis, storage and transportation as well as the receptors and enzymes involved in the binding, internalization, degradation and utilization of lipoproteins (Gwynne and Strauss, 1982; Golos et al., 1987b). The rate limiting step in the synthesis of P in granulosa cells is the side chain cleavage of cholesterol and formation of P_5. This irreversible reaction is regulated by gonadotropins (Hsueh et al., 1984; Golos et al., 1987a; Hsueh et al., 1989), estradiol (Toaff et al., 1983; Veldhuis et al., 1986; Keyes et al., 1990; Spicer et al., 1990), androgens (Welsh et al., 1982; Lee and Bahr, 1990), growth factors such as insulin-like growth factor (IGF-I; Adashi et al., 1985; Veldhuis et al., 1986; Urban et al., 1990), epidermal growth factor (EGF; Jones et al., 1982), platelet-derived growth factor and fibroblast growth factor (PDGF and FGF; Baird and Hsueh, 1980), and gonadotropin-releasing hormone (GnRH; Knecht et al., 1985; Kawai and Clark, 1986).

Immunofluorescent localization studies of cytochrome $P_{-450_{scc}}$ in ovarian tissues (Zlotkin et al., 1986), granulosa cells in culture (Goldring et al., 1986) and ovarian inner mitochondrial membranes (Farkash et al., 1986) have demonstrated marked differences in the amount of $P_{-450_{scc}}$ during various stages of follicular development. $P_{-450_{scc}}$ present in preovulatory follicles is greater than in small antral follicles, but is greatest in corpora lutea (Zlotkin et al., 1986). Granulosa cell P_{-450} mRNA levels are low in small follicles, but high in preovulatory ones. They rise markedly after the preovulatory LH surge or after human chorionic gonadotropin (hCG) treatment and are maintained at high levels in mature corpora lutea. Whereas the content of $P_{-450_{scc}}$ increases during luteinization, changes in the content of luteodoxin are minimal (Goldring et al., 1986).

Conversion of P_5 to P by 3β-HSD/Δ^{5-4} isomerase in the granulosa cells is regulated by gonadotropins, prolactin and androgens (Welsh et al., 1982). During P synthesis in rat granulosa cells cultured in the presence of gonadotropin, the activity of 3β-HSD is not a limiting factor. FSH stimulates the formation of P and 20α-OH-P, without

significant accumulation of P_5 (Dorrington et al., 1984). However, porcine granulosa cells cultured in the presence of gonadotropins accumulate more P_5 than P (Lischinsky et al., 1983).

P is the main active progestin produced by granulosa cells. Its secretion is modulated by its reversible conversion to the inactive metabolite, 20α-OH-P. Unlike P, 20α-OH-P cannot maintain pregnancy or decidual responses (Weist and Forbes, 1964) and an increase in 20α-OH-P is often associated with luteolysis (Hsueh et al., 1989). Conversion of P to 20α-OH-P is catalyzed by 20α-hydroxysteroid dehydrogenase (20α-HSD) which is believed to be regulated by gonadotropins (Weist et al., 1963; Hashimoto and Weist, 1969; Jones et al., 1983). Conversion of P to 20α-OH-P was observed in the rat (Weist et al., 1963), mouse (Loutfi et al., 1962), pig (Veldhuis, 1986, 1988) and human (Savard et al., 1965). However, in bovine corpus luteum the main metabolite of P is 20β-hydroxy-pregn-4-en-3-one (Savard et al., 1965).

3) Steroidogenesis in the Theca and Interstitial Cells

Even though the theca interna and interstitial cells have the ability to synthesize P and estrogens, the main steroid hormones produced by these cells are C_{19} androgens, including the 5-ene-3β-hydroxysteroids and 5α-reduced androgens. The primary steroidogenic precursor in theca cells is blood-born cholesterol delivered to cellular receptors in the form of lipoproteins. Theca cells also have the capacity for the de novo synthesis of cholesterol, and for the secretion of steroids synthesized from acetate (Ryan et al., 1968); however, steroidogenesis in the absence of lipoproteins is limited (MacLusky et al., 1981).

The major androgen synthesized by theca and interstitial cells is androstenedione. The more potent androgen, testosterone is produced in lesser amounts in the rat (Hamberger et al., 1978), cow (McNatty et al., 1984; Fortune and Hansel, 1985) and

human (McNatty et al., 1979; 1980) due to a deficiency in 17β-hydroxysteroid dehydrogenase.

Interstitial cells of the rat ovary contain 5α-reductases, capable of catalyzing 5α-reduction of C_{19} steroids and the production of androsterone and 5α-androstane-3α-17β-diol. Activity of 5α–reductase in the rat ovary is highest in preovulatory follicles (Eckstein, 1983) and decreases substantially during adulthood (Suzuki et al., 1978; Eckstein and Ravid, 1979) and in pubertal rats pretreated with pregnant mares' serum gonadotropin (PMSG) (Armstrong et al., 1975).

Theca cells can aromatize androgens to estrogens depending on the species and the stage of follicular development (Batta et al., 1980; Channing et al., 1978; Evans et al., 1981; Armstrong et al., 1981). In pigs (Evans et al., 1981) and sheep (Armstrong et al., 1981), thecal aromatase activities have been shown to increase with follicular growth and maturation. Porcine theca cells in culture also produce estrogen in the absence of exogenous androgen but estrogen synthesis by these cells does not appear to be regulated by gonadotropins (Tsang et al., 1982). Human theca cells produce only small quantities of estrogens, regardless of follicular size (Moon et al., 1978). P produced by the theca cells is mainly used for androgen biosynthesis. However, particularly in the human, 17α-hydroxyprogesterone is temporarily increased in the theca at midcycle and is the main hormone of corpus luteum. The primary metabolite of P in rat interstitial cells is 20α-dihydroprogesterone (Magoffin and Erickson, 1982).

C. Synthesis of Peptide Hormones and Other Nonsteroidal Factors in Granulosa Cells

In addition to steroid hormones, ovarian follicles secrete a variety of peptide hormones, prostaglandins, regulatory proteins, growth factors and mucopolysacharides. Many of these secretory products have an endocrine role outside the ovary. Others

function as autocrine or paracrine regulators, exerting their intra-ovarian effects on folliculogenesis, follicular cell differentiation (e.g. steroidogenesis) and ovulation.

1) Inhibin, Activin and Transforming Growth Factor-β (TGF_β)

One of the most extensively studied peptide hormones produced by granulosa cells is inhibin. Inhibin has been purified from follicular fluid (Robertson et al., 1985; Miamoto et al., 1985) and its cDNA has been cloned from porcine, bovine, human and rat ovaries (Mason et al., 1985; Robertson et al., 1985; Mayo et al., 1986; Woodruff et al., 1987). Inhibin is composed of two dissimilar subunits, α and β, which are linked together through cysteine disulfide bonds. There are two types of β subunits, called β_A and β_B. Homodimers of inhibin β subunits found in follicular fluid (Vale et al., 1986) are called either activin or FSH-releasing protein, since unlike inhibin, they have a stimulatory effect on FSH release from the pituitary (Ying, 1988). There is a striking homology between inhibin α, inhibin β (β_B dimer-activin) and transforming growth factor β (TGF_β), a molecule believed to be synthesized exclusively by theca-interstitial cells (Skinner et al., 1987) but recently found also in granulosa cell cultures (Kim and Schomberg, 1989). Dykema et al. (1991) have shown that inhibin and activin mRNAs are localized predominantly in the granulosa cells of ovarian follicles.

FSH, cAMP and forskolin increase inhibin secretion while androgens and estrogens are inhibitory (Dykema et al., 1991). La Polt et al. (1989) have shown recently that activin has a direct stimulatory effect on inhibin production and mRNA levels. Inhibin and activin act in an autocrine and paracrine manner to regulate follicular development; inhibin as an inducer of follicular growth and activin as an atretogenic factor (Woodruff and Mayo, 1990). Both peptides play an important role in the regulation of steroidogenesis.

TGF_β is produced by both theca and granulosa cells. Its mRNA has been detected in rat granulosa cells and large amounts of TGF_β are synthesized by cultured granulosa

cells (Kim and Shomberg, 1989; Hernandez et al., 1990 a). TGFβ has a mitogenic effect on granulosa cells. Through its inhibitory action on pituitary FSH release TGFβ attenuates LH receptor formation (Hutchinson et al., 1987). In addition, it also influences LH-induced maturation of oocytes (Tsafriri et al., 1989) and FSH-stimulated secretion of steroid hormones (Hutchinson et al., 1987).

2) GnRH-like Factor

GnRH-like proteins have been detected in rat granulosa cells (Sharpe, 1982; Oikawa, 1990) and in bovine, ovine (Aten et al., 1987) and human (Aten et al., 1989) ovaries. The ovarian protein which is physiochemically and immunochemically different from hypothalamic GnRH, was isolated from human follicular fluid (Li et al., 1987). In addition, GnRH mRNAs have been expressed in the granulosa cells from immature diethylstilbestrol-treated rats (Oikawa et al., 1990). Specific high affinity receptors for GnRH are present in the rat (Oikawa, 1990) and human (Li et al.,1987; Latouche et al., 1989) granulosa cells, although attempts to demonstrate ovarian GnRH receptors in sheep, cattle and pigs have failed. Direct physiologic effects of GnRH agonists have been reported in pig (Massicotte et al., 1980), cow (Milrae and Hansel, 1980), primate (Wickings et al., 1990) and rat (Leung and Wang, 1989) ovaries.

GnRH and GnRH-like factors are believed to play regulatory role in rat granulosa cell steroid production and differentiation. They have also been shown to induce ovulation in hypophysectomized rats by increasing the mRNA level and activity of tissue type plasminogen activator (Hsueh et al., 1988).

3) Prostaglandins

Prostaglandins are also secreted by granulosa cells. They are associated with and required for LH-induced ovulation in the rat, rabbit and pig (Espey, 1980). Prostaglandin content in porcine follicular fluid increases prior to ovulation (Ainsworth et al., 1975;

Tsang et al., 1979). Prostaglandin E and $F_{2\alpha}$ synthesis is elevated significantly in rat preovulatory follicles but not in small follicles incubated with LH or FSH (Richards and Bogovich, 1982). It is not clear what biochemical and hormonal mechanisms are involved in the regulation of prostaglandin synthesis in follicles at different developmental stages and at specific times after the LH surge. Indirect evidence suggests the importance of arachidonic acid (AA) availability (Tsang et al., 1988) and an increase in prostaglandin synthase (PGS, catalyzing the conversion of AA to prostaglandins E, $F_{2\alpha}$ and thromboxane A_2), and prostacyclin synthase [ISN, converting PGH_2 to prostacyclin (PGI_2)] (Smith, 1986; Richards, 1987). HCG-induced PGS is localized primarily in granulosa cells, while ISN is found mainly in theca cells (Richards, 1987). The induction of PGS is transient. The enzyme reaches maximal level prior to ovulation and begins to decrease thereafter (Hedin et al., 1987).

Prostaglandins stimulate plasminogen activator, proteoglycan and oxytocin production by granulosa cells (Strickland and Beers, 1976; Wang and Leung, 1982; McArdle and Holtorf, 1989). Inhibitors of prostaglandin synthesis are also effective inhibitors of ovulation (Espey, 1980, Strickland and Beers, 1976; Le Maire et al., 1973) supporting the notion that prostaglandins play an important role in the follicular rupture during the ovulatory process.

4) Insulin-like Growth Factors (IGFs)

IGFs exhibit extensive structural and functional similarities to insulin. Insulin consists of a single chain with two subunits (A and B) joined by disulfide bonds. It has a connecting C peptide that is cleaved from the native hormone upon release. More than 45% of the amino acid sequence of the A and B chains of the IGFs are identical to those of the insulin molecule but C and D chains of the IGFs are unique (Hsueh et al., 1989). Like insulin, IGFs are secreted in the form of a large, inactive precursor. IGFs and insulin can share cellular receptors; however, specific IGF-I and IGF-II receptors have been

identified in theca and granulosa cells (Morgan et al., 1987). Both Type I and Type II IGF receptor genes are expressed in granulosa and theca-interstitial cells (Hernandez et al., 1990 b).

FSH and estrogens stimulate IGF-I production by porcine granulosa cells in vitro (Hsu and Hammond, 1987) and growth hormone is also stimulatory in hypophysectomized rats in vivo (Davoren et al., 1986). In the rat ovary IGF-I gene expression has been localized exclusively in granulosa cells (Murphy et al., 1987; Hernandez et al.,1989). IGF-I appears to play an important role as an intraovarian modulatory signal. Its autocrine and paracrine actions in the regulation of steroidogenesis will be discussed in the next section.

III. REGULATION OF OVARIAN STEROIDOGENESIS

A. Role of FSH

Follicle-stimulating hormone (FSH) is essential for the development of ovarian follicles. It is the prime inducer of granulosa cell maturation and it accounts for the responsiveness of the cells to other hormones and regulatory factors. FSH stimulates granulosa cell secretion of estrogens and progestins as well as of many nonsteroidal substances.

1) FSH Structure

FSH is a glycoprotein hormone produced by the anterior pituitary. Like LH and thyroid stimulating hormone (TSH), it contains two noncovalently linked dissimilar subunits, α and β. The α subunit is identical for these three hormones within a species and it is the unique β subunit that confers biological specificity (Hsueh et al., 1989). The protein domain of the glycoprotein binds to FSH receptors on granulosa cells; the

carbohydrate moiety interacts with membrane components, probably within the receptor molecule. It has been suggested that the carbohydrate side-chains of FSH interact with putative cell membrane lectins to allow the coupling of the hormone-receptor complex to adenylate cyclase. The binding of both components of FSH to the cellular receptors leads to signal transduction through the activation of intracellular second messenger systems.

2) FSH Receptors

FSH binding to granulosa cells is detectable even in primary follicles, when the oocytes cease to grow (Midgley, 1973, Richards and Midgley, 1976). In the rat, it is measurable by the end of the first week of life (Peluso et al., 1976) and increases during follicular development to a maximum at 28 days (White and Ojeda, 1981). Within the follicular complex, FSH binds exclusively to granulosa cells. Autoradiographic studies with [^{125} I] FSH have revealed that FSH receptors are distributed in all granulosa cell layers of preovulatory follicles (Midgley, 1973). The factors which control the appearance of FSH receptors in vivo are not completely understood. Depending on the experimental conditions, FSH has been shown to both up- and down-regulate its own receptors in granulosa cells in vitro (Richards et al., 1976; Ireland and Richards, 1978). Estrogens synergise with FSH to increase the number of receptors for this gonadotropin (Richards et al., 1976; 1987). However there is also evidence that the preovulatory LH surge decreases the receptors content of both gonadotropins (Richards, 1980).

The FSH receptor is a single 75 kD polypeptide with a 348 amino acid residue extracellular domain which contains three N-linked glycosylation sites. This domain is connected to a structure containing seven putative transmembrane segments with sequence similarity to G protein-coupled receptors (Sprengel et al., 1990). FSH receptors display substantial structural and sequential similarity to LH receptors (Segaloff et al., 1990). According to Zhang and co-workers (1988), FSH receptors are physically and functionally coupled with G proteins. Occupancy of guanosine triphosphate (GTP)

binding sites on G protein coupled to FSH receptors is necessary for the GTP effect on FSH-receptor interaction (Zhang et al., 1988, 1991). The stimulatory G protein (G_S) has been suggested to be the protein important for the regulation of FSH-receptor binding by GTP (Zhang et al., 1991).

3) Regulation of Progestin Biosynthesis

The production of P and its metabolites is one of the main biosynthetic activities of granulosa cells. It occurs initially in response to FSH stimulation, but is augmented by LH in later stages of follicular development. The influence of LH on cultured granulosa cells from hypophysectomized estrogen-treated rats is evident only after FSH priming (to induce LH receptors) in vivo (Miller et al., 1978) or in vitro (Wang et al., 1981). FSH but not LH stimulates P biosynthesis in undifferentiated granulosa cells (Dorrington and Armstrong, 1979; Hsueh, et al., 1984).

The effect of FSH on granulosa cell progestin production involves an increase in cholesterol delivery through the stimulation of lipoprotein (LDL, HDL) binding, internalization and degradation as well as de novo cholesterol biosynthesis. FSH increases the association of cholesterol with the $P_{-450_{scc}}$ enzyme complex via a low-molecular weight activator peptide, and the supply of the intra-mitochondrial cholesterol via a cytoskeleton-dependent process (Hall, 1984).

FSH stimulates granulosa cell progestin biosynthesis also by modulating the activities of various steroidogenic enzymes (Fig. 3). One of the main regulatory sites of FSH action in granulosa cells is the cholesterol side-chain cleavage (Jones and Hsueh, 1982; Toaff et al., 1983). Addition of 25-hydroxycholesterol enhances the stimulatory effect of FSH on P production, implying a role of this gonadotropin in increasing cholesterol side-chain activity (Jones and Hsueh, 1982). Trzeciak et al., (1986) have demonstrated that FSH induces the synthesis of all three components of the rate limiting enzyme in rat granulosa cells: cytochrome ($P_{-450_{scc}}$), iron sulphur protein (ISP) and

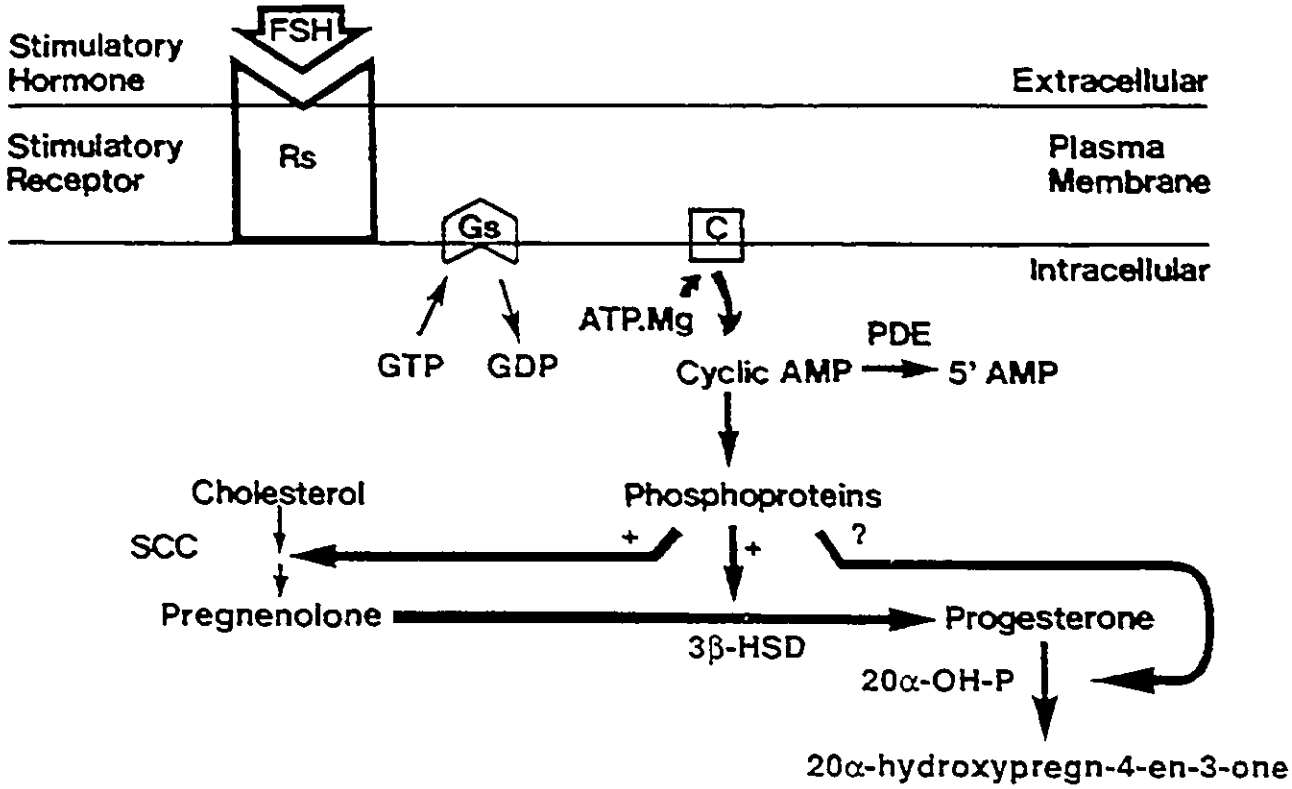

Figure 3 Mechanism of FSH action in the regulation of steroidogenic enzymes in granulosa cells. [Modified after Hsueh et al. (1984) and Roth and Grunfeld, (1985)].

R_S	- stimulatory receptor
G_S	- stimulatory G protein
C	- catalytic subunit of adenylyl cyclase
PDE	- phosphodiesterase
SCC	- cholesterol side chain cleavage enzymes
3β-HSD	- 3β-hydroxysteroid dehydrogenase
20α-HSD	- 20α-hydroxysteroid dehyrogenase

NADPH: ISP reductase (Red). The induction is coordinated, dose- and time-dependent and involves increased levels of translatable mRNA encoding the precursor forms of $P_{-450_{scc}}$ and ISP. These effects of FSH are probably cAMP-mediated (Trzeciak et al., 1986). The obligatory role of FSH for the induction and maintenance of $P_{-450_{scc}}$ synthesis has been confirmed by Goldring et al. (1986). Granulosa cells adjacent to the basement membrane (mural cells) respond better to FSH than cells close to the oocyte (cumulus cells). A few layers of cumulus granulosa cells seem to lack the enzyme at all stages of follicular development. These observations are in accord with the findings by Kasson et al. (1985), demonstrating variable response of subpopulations of granulosa cells in immature rats to stimulation by FSH.

Granulosa cell 3β-HSD/ Δ^{5-4} isomerase activity is also regulated by FSH (Zeleznik et al., 1974). Treatment of rat granulosa cells in vitro with the gonadotropin significantly increases the conversion of P_5 to P (Jones and Hsueh, 1982). The reaction is irreversible and apparently not rate limiting for P synthesis (Dorrington et al., 1984) (Fig. 3).

The effect of FSH on 20α-HSD, which converts P to its less active metabolite 20α-OH-P appears to be equivocal in rat granulosa cells. Induction of 20α-HSD by FSH has been demonstrated in hypophysectomized, estrogen treated rats (Eckstein and Nimrod, 1979), although the influence of the gonadotropin in vitro has been variable (Jones and Hsueh, 1981; Welsh et al., 1984, Kawai and Clark, 1986) (Fig. 3).

Granulosa cells contain all the enzymes necessary for the conversion of cholesterol to progestins, but are unable to synthesize androgens necessary for estrogen production. Androgens synthesized by theca-interstitial cells are used by granulosa cells for estrogen biosynthesis, and much of the theca interna cell-derived androgens are produced from P supplied by granulosa cells (Hsueh et al., 1989). LH stimulates the biosynthesis of androgens in the theca compartment. Androgens are converted to estrogens in granulosa cells under the influence of FSH (Dorrington and Armstrong, 1979; Moon et al., 1975).

Dorrington et al. (1975) first demonstrated the ability of FSH to induce aromatase in rat granulosa cells in vitro. The stimulatory influence of FSH was later confirmed in vivo in hypophysectomized rats (Armstrong and Papkoff, 1976). Studies using antibodies directed against purified aromatase P_{-450} have shown that FSH increases the production of this enzyme in cultured granulosa cells (Steinkampf et al., 1987) Although aromatase activity is mainly stimulated by FSH, in vitro studies have shown that LH may also increase estrogen production in granulosa cells from FSH-primed rats (Erickson et al., 1979; Wang et al., 1981).

4) Regulation of Biosynthesis of Nonsteroidal Factors

In granulosa cells, FSH stimulates the secretion of a variety of nonsteroidal substances, including those involved in the process of ovulation. In vitro studies have shown that FSH increases the production of prostaglandins (PGE and $PGF_{2\alpha}$) (Richards, 1987; Ainsworth et al., 1990) and of plasminogen activator (Ny et al., 1985; Canipari and Strickland, 1985, 1986). It also stimulates the incorporation of [^{35}S] sulfate into ovarian glycosaminoglycans (GAGs) (Muller et al., 1978) and increases follicular fluid levels of proteoglycans (Adashi et al., 1986). Ovarian oxytocin (Schams, 1987), renin and angiotensin levels are also elevated by the gonadotropin and the content of renin mRNA is increased by FSH and estradiol (Kim et al., 1987 b). In addition, inhibin production is significantly higher in the presence of FSH (Biscak et al., 1986).

5) Effect of FSH on Proliferative Activity and Cellular Morphology of Granulosa Cells.

One of the actions of FSH is its stimulation of mitosis, which increases granulosa cell numbers in the developing Graafian follicle (McNatty et al., 1979). FSH enhances granulosa cell protein synthesis (Ahren and Rubinstein, 1965; Ahren et al., 1967) and carbohydrate metabolism (Hamberger and Ahren, 1967; Farmer et al., 1973). The

stimulatory effect of FSH on follicular growth depends on its interaction with estradiol and is influenced by a variety of growth factors (Erickson, 1986).

FSH induces a number of membrane-related phenomena. It enhances the development of microvilli, a primary site of LH receptors (Amsterdam and Rotmensch, 1987) and the formation of LH, prolactin, lipoprotein and EGF receptors (Erickson, 1986; Amsterdam and Rotmensch, 1987; Freeman, 1988). The ability of FSH to induce LH receptors is specific to the granulosa cells of dominant, preovulatory follicles (Erickson, 1986).

FSH has marked effects on the morphology of granulosa cells. It causes flat epithelioid cells in culture to round up and assume a nearly spherical shape (Amsterdam et al., 1981). This transformation appears similar to the changes observed in granulosa cells during the development of primary to secondary follicle in vivo. In addition, the number and size of gap junctions increase significantly after in vivo or in vitro exposure to FSH (Amsterdam et al., 1981; Burghardt and Matheson, 1982). The enhancement of steroidogenesis by this gonadotropin is accompanied by significant changes in cellular organelles. Rough endoplasmic reticulum is gradually replaced by developing, tubular smooth endoplasmic reticulum. Mitochondria in granulosa cells shift from lamellar ones in immature granulosa cells to a highly complex, tubular type in mature follicles. Once a granulosa cell has been exposed to FSH, its survival becomes dependent upon the continual presence of the gonadotropin (Erickson, 1986; Amsterdam and Rotmensch, 1987).

B. Role of LH

LH stimulates follicular steroidogenesis, induces ovulation and is involved in corpus luteum formation and maintenance. It also increases the secretion of nonsteroidal substances and modulates plasma membrane hormonal receptor content in ovarian cells. In addition, LH influences granulosa cell morphology and regulates their overall functions.

1) LH Structure

LH belongs to the same family of pituitary glycoproteins as FSH and TSH. It contains two noncovalently linked dissimilar subunits α and β, of which α subunit is common to all three hormones and the β subunit is hormone-specific (Hsueh et al., 1989).

2) LH Receptors

LH receptors are present on granulosa cells at the time of antrum formation. The presumptive theca cells acquire their LH receptors shortly after the cells reach the stage of the secondary follicle (Erickson et al. 1985). Several in vivo and in vitro studies have demonstrated the obligatory role of FSH in LH receptor induction (Hsueh et al., 1984; Amsterdam and Rotmensch, 1987). It is noteworthy that this action of FSH is selective to the granulosa cells of dominant preovulatory follicles. Withdrawal of FSH results in the loss of both FSH and LH receptors and death of the granulosa cells (Richards, 1980; Erickson, 1986). Estradiol and small increases in serum LH that precede the LH surge also seem to be obligatory for the induction of LH receptors on granulosa cells of preovulatory follicles (Bogovich et al., 1981; Richards, 1980; Richards and Bogovich, 1982). Progestins (Rami et al., 1981), androgens (Rami et al., 1981), insulin (May et al., 1980) and IGF-I (Adashi et al., 1985) enhance the induction of LH receptors by FSH in cultured granulosa cells. On the contrary, the LH surge, EGF (Mondschein and Schomberg, 1981) and glucocorticoids (Erickson et al., 1983) inhibit the stimulatory effect of FSH on LH receptor content (Richards, 1980).

Most of the LH receptors are located on the microvillar area of the cell surface (Amsterdam et al., 1981; Amsterdam and Lindner, 1984) and exist in monomeric form or in clusters. The formation of clusters appears to be associated with down-regulation of the receptors (Amsterdam and Rotmensch, 1987). The number of LH receptors on the granulosa cell varies depending on its follicular localization. Mural granulosa cells have 7

to 10 times more LH receptors than cumulus cells (Amsterdam and Lindner, 1984). Upon binding to its receptor, LH stimulates adenylate cyclase coupled to G_S protein. The granulosa cell LH receptor is a single chain. It possesses a large extracellular domain responsible for hormone binding and a region that spans the plasma membrane seven times. The peptide has a relatively short intracellular c-terminal tail which is probably involved in coupling with the G_S protein (Segaloff, 1990).

3) Regulation of Steroid Biosynthesis

LH is an important regulator of androgen production in theca-interstitial cells. It also increases progestin and estrogen synthesis in luteinized granulosa cells, luteal cells and theca-interstitial cells. The ability of LH to stimulate steroid synthesis involves both acute and chronic mechanisms. Acute stimulation occurs within few minutes and involves a second messenger system which induces alterations in cholesterol metabolism (Erickson et al., 1985; Ghosh et al., 1987). HCG has been shown to increase binding, internalization and degradation of LDL in luteinized human granulosa cells (Soto et al., 1984; Golos et al., 1985). In the absence of adequate exogenous steroidogenic substrate, LH stimulates HMG-CoA reductase to increase de novo cholesterol synthesis (Rodgers et al., 1987b) and induces cholesterol esterase activity for the mobilization of cholesterol from lipid droplets (Caffrey et al., 1979).

The long-term control by LH is at the level of gene transcription of key steroidogenic enzymes (Golos et al., 1985; Ghosh et al., 1987). A primary site of action of LH on steroid synthesis in ovarian cells is the rate-limiting step, cholesterol side-chain cleavage. Via cAMP as a second messenger, LH increases the capacity of the $P_{-450_{scc}}$ to metabolize cholesterol to P_5. It also enhances the amount of cholesterol specifically bound to $P_{-450_{scc}}$ by increasing cholesterol transfer from lipid droplets to mitochondria and into the inner mitochondrial membrane (Erickson et al., 1985; Golos et al., 1987a; Ghosh et al., 1987).

Another important step regulated by LH in granulosa, theca-interstitial and luteal cells is the conversion of P_5 to P. HCG has been shown to increase 3β–HSD activity and 3β-HSD mRNA expression in porcine (Chedrese et al., 1990a) and rat (Madej, 1980; Jones et al., 1983) granulosa cells. The role of LH in the regulation of 20α-HSD is unclear since the effects of the gonadotropin on the activity of enzyme have been variable (Eckstein et al., 1977; Hsueh et al., 1984; Kawai and Clark, 1986). In the rat, a large preovulatory peak of P and 20α-OH-P is present just prior to the LH surge and is probably due in part to LH receptor induction. The primary cellular source of progestins probably constitute the luteinized granulosa cells, however the contribution of the theca cells is also significant (Gore-Langton and Armstrong, 1988). In the contrast, P production and hormone responsiveness are highest in cultured bovine theca cells from small follicles and lowest in those from large follicles (Roberts and Skinner, 1990).

Ovarian androgen production in vivo depends on the circulating concentration of LH. Small increases in serum LH stimulate androgen production although the LH surge is inhibitory (Richards and Hedin, 1988), due in part to its biphasic effects on $P_{-450_{17\alpha}}$ gene transcription (Hedin et al., 1987; Rodgers et al., 1987a). The decline in androgen production in theca-interstitial cells induced by LH does not appear to be due to an inability of the cells to synthesize these hormones, since theca cells produce androgens in a constitutive manner in the absence of the gonadotropin (Richards et al., 1986).

The induction of LH receptors by FSH in the granulosa cells in late stage of follicular development renders the cells capable of responding to LH and of maintaining aromatase activity. Increased concentrations of estradiol augment LH receptor induction (Richards, 1980; Richards and Hedin, 1988). Small increases in serum LH and enhanced androgen production are key factors necessary for the initial increase in estradiol biosynthesis in small antral follicles of the rat (Richards and Hedin, 1988). Following the LH surge, estradiol biosynthesis declines, probably due to a decrease in androgen

production (Eckstein and Tsafiriri, 1986), although, there is also evidence for an inhibitory action of LH on the aromatase enzyme (Hedin et al., 1987).

4) Effect of LH on Hormonal Receptors

The responsiveness of ovarian cells to LH depends on its concentration in serum and on the abundance of its cellular receptors. Low concentrations of LH increase the content of its own receptors, but the LH surge decreases both LH and FSH receptor numbers (Richards, 1980). In contrast, the LH surge increases the content of prolactin (Richards, 1980), β-adrenergic and lipoprotein receptors on granulosa cells (Amsterdam et al., 1981; Amsterdam, 1984; Hsueh et al., 1984).

5) Effect of LH on Ovarian Production of Nonsteroidal Substances and on Cell Morphology

Ovulation is initiated by the LH surge through its stimulatory effect on the biosynthesis of prostaglandins (Richards, 1987) and the plasminogen activator system (O'Connel et al., 1987). Prostaglandins and P are important for the control of follicle rupture. Granulosa cell tissue-type plasminogen activator (t-PA) activity and t-PA mRNA level increase significantly before ovulation. Other cellular events associated with the action of LH prior to ovulation include alterations in granulosa cell shape and reduction in the number of granulosa-granulosa and granulosa-oocyte gap junctions, resumption of meiotic maturation and the expansion of the oocyte-cumulus complex (Hsueh et al., 1984).

One of the important actions of LH on ovarian follicular cells is that related to luteinization. The luteotropic effect of LH is partially expressed through its stimulatory influence on prolactin receptor content (Richards, 1980). Luteinization involves the development of smooth endoplasmic reticulum and biochemical as well as morphological changes in the mitochondria, including an increased complexity of their cristae and

$P_{-450_{scc}}$ levels. In addition, elevated amounts of lipid droplets which contain cholesterol are observed during luteinization (Gulyas, 1984).

6) Effect of LH on General Cell Functions

Like FSH, LH increases glucose uptake and stimulates transcriptional and translational processes in granulosa cells, leading to increased production of specific proteins essential for steroidogenesis. DNA synthesis is stimulated by LH in granulosa cells from preovulatory follicles, but is inhibited in cells undergoing luteinization (Hsueh et al., 1984).

C. Role of Prolactin

Prolactin is the primary luteotropic stimulus for the maintenance of luteal functions (Gulyas, 1984). In rodents and several other mammalian species, the luteotropic action of prolactin seems to be indispensable, particularly in early pregnancy and pseudopregnancy (Richards, 1980). Prolactin is involved in the luteinization of granulosa cells which is accompanied by increased complexity of mitochondrial structure, presence of smooth endoplasmic reticulum, cell rounding (Fortune and Vincent, 1986) as well as decreased P catabolism (Rotchild, 1981).

Prolactin stimulates P biosynthesis in rat granulosa cells in vitro (Rotchild, 1981; Fortune and Vincent, 1986; Peluso, 1989). In swine, however, it inhibits granulosa cell P biosynthesis in small immature follicles but stimulates that in mature ones (Veldhuis et al., 1980). The appearance of specific prolactin receptors in granulosa cells in late follicular development and their induction by FSH in vitro (Zhuang et al., 1982) suggests a physiologic role of the hormone in granulosa cell differentiation and transformation to luteal cells (Gore-Langton and Armstrong, 1988). Prolactin is known to increase and

maintain LH receptor content, (Gibori and Richards, 1978; Advis et al., 1981) and to augment LH-stimulated adenylyl cyclase activity (Day and Birnbaumer, 1980).

Prolactin inhibits FSH-induced estrogen production by granulosa cells of preantral and antral follicles in vivo and in vitro (Fortune et al., 1986b). This inhibitory action of prolactin appears to be expressed at the level of aromatase enzyme. Recent studies have confirmed that the hormone specifically inhibits the expression of aromatase mRNA in rat granulosa cells before and during the early stage of luteinization (Krasnow et al., 1990). During early gestation prolactin also causes a reduction in luteal aromatase activity but in later stages it is stimulatory (Hickey et al., 1989).

In addition, prolactin attenuates LH-induced androgen biosynthesis in theca-interstitial cells (Erickson et al., 1985), although it is ineffective in ovarian explants in vitro (Hedin et al., 1987). Prolactin inhibits granulosa cell estrogen and theca-interstitial cell androgen synthesis at a step(s) distal to cAMP production (Gore-Langton and Armstrong, 1988).

D. Role of Thyroid Hormones and Glucocorticoids

Thyroid hormones and glucocorticoids appear to play significant roles in the regulation of granulosa cell function. Ovarian follicular development and luteinization induced by gonadotropins are attenuated in thyroidectomized animals(Gore-Langton and Armstrong, 1988). Channing et al. (1976) have shown that thyroid hormones and insulin increase luteinization in cultured porcine granulosa cells. Thyroxine and triiodothyronine augment FSH-stimulated LH receptor induction and P and estradiol secretion in porcine granulosa cells of small but not of medium or large follicles in vitro (Maruo et al., 1987).

Glucocorticoid receptors and cortisol binding globulin are present in rat granulosa cells (Louvet et al., 1977) and in porcine follicular fluid (Mahajan and Little, 1980) respectively. In addition, cortisol and dexamethasone enhance FSH-stimulated P synthesis

(Adashi et al., 1981) but inhibit aromatase activity induced by the gonadotropin (Hsueh and Erickson, 1978). The effect of glucocorticoids on P production in granulosa cells appears to be due to increased 3β-HSD and decreased 20α-HSD activities.

E. Paracrine and Autocrine Control of Steroidogenesis

Considerable attention has been focused recently on factors which regulate follicular cell functions in a paracrine or autocrine manner. These factors including peptides, steroids and cytokines, are produced locally in the ovary and play an important regulatory role in granulosa cell steroidogenesis and folliculogenesis. They regulate the synthesis of various biological substances in the theca and granulosa cells and modulate the follicular responses to gonadotropins. Receptors for many of these regulators have now been identified in ovarian cells (Hsueh et al., 1984; Amsterdam and Rotmensch, 1987; Hsueh et al., 1989).

1) Role of Estrogens

Estrogens stimulate follicular growth and development through their action on granulosa cell proliferation, formation of gap junctions, and follicular antrum formation (Hsueh et al., 1984; Hillier and Turner, 1990). They also prevent follicular atresia (Richards, 1975; Terranova, 1981). In addition, estrogens enhance FSH-induced LH receptor content (Hillier and Turner, 1990), synergize with FSH in the induction and maintenance of FSH receptors and increase the content of their own receptors on granulosa cells (Richards et al., 1987).

Estrogens modulate basal and gonadotropin-stimulated steroid hormone secretion in granulosa and theca cells. Studies on rat luteal cells have shown that estradiol stimulates lipoprotein hydrolysis, de novo synthesis of cholesterol, HMG-CoA reductase activity and cholesteryl ester turnover (Azhar et al., 1989). In addition, it increases the

number of LDL receptors, LDL metabolism and LDL -induced P production in swine granulosa cells (Veldhuis and Gwynne, 1985). Estrogens have variable effects on follicular androgen and progestin production, depending on their concentration, the species examined and the stage of ovarian follicular development (Lee and Bahr, 1989; Keys et al., 1990). Estradiol decreases LH-stimulated androgen production in rat (Leung and Armstrong, 1979; 1980) and swine (Leung and Armstrong, 1980; Hunter and Armstrong, 1986) theca cells in vitro but increases that in bovine cells (Roberts and Skinner, 1990). It was suggested that a local feedback loop exists in ovarian follicles where androgen produced by thecal cells of small follicles is used as a substrate for estrogen production by granulosa cells. Estrogen in turn stimulates androgen production in the thecal cells (Roberts and Skinner, 1990). Roberts and Skinner (1990) hypothesized that thecal androgen synthesis is stimulated by estradiol until estrogen biosynthesis is interrupted, as in the case of atresia, or when the concentration of estradiol is greater than 1μM (i.e. preovulatory estradiol surge). Estradiol appears to act at a site distal to cAMP production and probably at an enzymatic step(s) in the steroidogenic pathway between androgens and their C_{21} precursors (Gore-Langton and Armstrong, 1988).

Estrogen is known to regulate its own production. It increases basal and gonadotropin-stimulated aromatase activity (Zhuang et al., 1982; Hillier and Turner, 1990) Concomitant treatment of granulosa cells with FSH and estrogen enhances the stimulatory effect of the gonadotropin on estradiol synthesis (Adashi and Hsueh, 1982; Zhuang et al., 1982).

The influence of estrogens on P production by granulosa and thecal cells is variable. Estrogens dose-dependently decrease P secretion in porcine and bovine thecal and granulosa cells (Fortune and Hansel, 1979; Hunter and Armstrong, 1987; Roberts and Skinner, 1990), and human luteal cells (Tonetta et al., 1987). An increase in P_5 synthesis (Fortune, 1986) and a decrease in P production (Roberts and Skinner, 1990) have been observed in thecal cells of medium and large bovine follicles. Estrogens have also been

shown to stimulate P secretion by porcine (Veldhuis et al., 1982) and rat (Hillier et al., 1977; Welsh et al., 1983) granulosa cells and porcine thecal cells from small follicles (Roberts and Skinner, 1990). Likewise gonadotropin-induced progestin biosynthesis in granulosa cells from immature, hypophysectomized rats is increased by estrogen (Welsh et al., 1983). Although estradiol decreases 3β-HSD activity in porcine thecal cells (Tonetta et al., 1987) and hen granulosa cells (Lee and Bahr, 1989) it increases cytochrome $P_{-450scc}$ activity and content (Toaff et al., 1983; Veldhuis and Gwynne, 1985; Spicer et al., 1990; Hillier and Turner, 1990).

2) Role of Androgens

Androgens produced by thecal and interstitial cells are obligatory intermediates for estrogen biosynthesis (Hsueh et al., 1984; Gore-Langton and Armstrong, 1988). They also play an important role in the autocrine and paracrine regulation of the development and function of the ovary. The actions of androgens on follicular maturation are antagonistic. In contrast to estrogens (which promote follicular development) androgens induce follicular atresia, decrease estrogen-induced ovarian weight gain and eventually promote the death of the granulosa cells and of the ovum (Payne and Rusner, 1958; Hillier and Ross, 1979). Atresia is accompanied by a decrease in granulosa cell LH and estrogen receptors (Farookhi, 1982; Saiduddin and Zassenhaus, 1978). A high androgen/estrogen ratio is an inevitable sign of nonovulatory follicles and atresia (Carson et al., 1981).

Androgens have been shown to either stimulate or inhibit P production, depending on the species and stage of follicular development . In vitro, androgens stimulate P biosynthesis in granulosa cells from rat, porcine and mouse preantral follicles (Hsueh et al., 1984; Gore-Langton and Armstrong, 1988). This response does not appear to be due to the action of estrogen accumulated from androgen metabolism, since both aromatizable and nonaromatizable androgens are effective in stimulating P synthesis. Androgens have been shown to enhance FSH-stimulated P secretion by rat granulosa cells (Armstrong and

Dorrington 1976; Welsh et al., 1982). This effect can be blocked by the antiandrogen hydroxyflutamide (Hillier and de Zwart, 1982). Recent studies by Hillier and Turner (1991) have shown that androgens increase cAMP accumulation and expression of genes encoding FSH-inducible proteins including $P_{-450scc}$ and inhibin and activin subunits.

In contrast androgens have been reported to inhibit basal P production by human granulosa cells (Batta et al., 1980) and gonadotropin-induced P accumulation by porcine (Lischinsky et al., 1983; Evans et al., 1984) and hen (Johnson et al., 1988) granulosa cells. At least in granulosa cells from prepubertal pig, androgen appears to have a direct inhibitory action on P synthesis but not metabolism (Lischinsky et al., 1983; Evans et al., 1984). Although there is a general agreement on the stimulatory influence of FSH and testosterone on the activity of $P_{-450scc}$ (Jones and Hsueh, 1982, Welsh et al., 1982; Hillier and Turner, 1990), the effect of androgens on 3β-HSD/Δ^{5-4} isomerase is equivocal. Whereas some data suggest that androgens act synergistically with FSH to increase conversion of P_5 to P (Welsh et al., 1982), other indicate that androgens are either ineffective at this site (Dorrington and Armstrong, 1979) or suppress P production by inhibiting 3β-HSD activity (Tan and Armstrong, 1984). In addition to serving as substrate for estrogen biosynthesis, androgens augment gonadotropin-stimulated aromatase activity in cultured granulosa cells (Hillier and de Zwart, 1981).

3) Role of Progestins

Specific P receptors have been identified in the ovarian cells of the rat (Naess, 1981; Schreiber et al., 1983) guinea pig, rabbit, human (Hsueh et al., 1984; Gore-Langton and Armstrong, 1988) and nonhuman primate (Stouffer, 1991). Although ovarian follicles secrete large quantities of P the role of this steroid in the regulation of ovarian follicular development and function is unclear. It has been suggested that the action of P in vivo may be due to its effect on pituitary secretion of gonadotropins (Beattie and Corbin, 1975).

P is known to regulate its own secretion. The synthetic progestin R5020 increases FSH-stimulated P and 20α-OH-P synthesis in granulosa cells from hypophysectomized, estrogen-treated rats. It also augments LH-induced progestin production by preovulatory cells primed with FSH. P has been shown to enhance cAMP production by rat granulosa cells in response to FSH (Goff et al., 1979) and to increase gonadotropin-induced P_5 synthesis (Fanjul et al., 1983) in vitro.

Progestins decrease ovarian aromatase activity and the biosynthesis of estrogens. Fortune and Vincent (1983) have shown that P has a specific and irreversible inhibitory effect on estradiol production by granulosa cells in vitro. The reduction of FSH-induced estrogen secretion in granulosa cells of immature hypophysectomized, diethylstilbestrol-treated rats by P, 20α-OH-P, or R5020 in vitro has also been reported (Schreiber et al., 1980). In addition, the inhibitory effect of progestins has been demonstrated in vivo (Saidapur and Greenwald, 1979). Administration of P to proestrous hamsters decreases serum estradiol concentration without any change in the circulatory levels of gonadotropins. Since injection of aromatizable androgens fails to reverse the inhibitory influence of P, it is possible that the action of the progestin may be at the level of the aromatase enzyme and not at the level of synthesis of androgen precursors. Greenwald (1974) suggested that the inhibitory effect of P is one of the factors responsible for the sharp decline in estrogen secretion following the LH surge in hamsters. It has also been proposed that P plays a role in the selection of the dominant follicle. The less differentiated the granulosa cells, the more pronounced is the inhibitory effect of this steroid. It seems that, through increased P secretion, the dominant follicle maintains its neighboring follicles in an immature state by inhibiting their aromatization potential (Franchimont et al., 1988).

4) Role of Gonadotropin-Releasing Hormone-Like Peptides

GnRH and GnRH-like peptides have both stimulatory and inhibitory effects on granulosa cell steroidogenesis. GnRH and its analogues increase basal P_5, P and 20α-OH-P production by granulosa cells from intact and immature hypophysectomized estrogen-primed rats (Jones and Hsueh, 1982a; Jones and Hsueh, 1982b; Dorrington et al., 1984). GnRH is known to stimulate $P_{-450scc}$ (Jones and Hsueh, 1982; Dorrington et al., 1984), 3β-HSD/Δ^{5-4} isomerase (Dorrington et al., 1983; Jones and Hsueh, 1981), 20α-HSD (Jones and Hsueh, 1981) and aromatase (Dorrington et al., 1983) activities. The direct effects of GnRH and GnRH-like substances are much weaker than the stimulatory action of FSH and seem to depend on the duration of hormonal exposure and on the stage of follicular maturation (Raani et al., 1983; Popkin et al., 1983).

In primary cultures of rat granulosa cells GnRH and its agonists suppress FSH-induced progestin and estrogen biosynthesis (Jones and Hsueh, 1982; Jones et al., 1983, Welsh et al., 1984; Knecht et al., 1985; Wang et al., 1989). P production by granulosa cells from human preovulatory follicles aspirated after hCG administration is also sensitive to GnRH agonist (Parinaud et al., 1988). The inhibitory action of GnRH seems to be due to the suppression of FSH action on $P_{-450scc}$ and 3β-HSD activities and the stimulation of 20α-HSD (Jones and Hsueh, 1981). The latter leads to a reduction of P secretion with concomitant increase in 20α-OH-P accumulation. LH- or hCG-stimulated estrogen and P production is suppressed by GnRH in rat granulosa cells primed with FSH (Jones and Hsueh, 1981; Jones et al., 1984). GnRH has also been shown to suppress the 17α-hydroxylase: C-17,20-lyase enzyme activities and the LH-induced androgen production by theca-interstitial cells in vitro (Magoffin and Erickson, 1982).

Direct inhibitory action of GnRH and its analogs on rat granulosa cell differentiation includes decreased steroidogenesis, impaired cAMP production and reduction in gonadotropin receptor expression (Hsueh and Jones, 1981; Knecht et al., 1985). An uncoupling of the FSH receptor from adenylate cyclase and a loss of FSH

receptors have been described following treatment of rat granulosa cells with GnRH agonist (Knecht et al., 1983). A decrease in LH, prolactin and EGF receptors has been reported in granulosa cells and luteal cells cultured with GnRH (Hsueh and Ling, 1979; Hsueh et al., 1980; Hsueh and Jones, 1981; St. Armand et al., 1983).

GnRH is believed to act through a cAMP-independent signalling system (Davis et al., 1986; Ekstein et al., 1986). Binding of GnRH to its membrane receptors activates phosphatidylinositol pathway which leads to increases in the intracellular Ca^{2+} concentration and the activation of protein kinase C (PKC), with the latter playing an important modulatory role in the control of steroid secretion by granulosa cells (Leung and Wang, 1989). In addition, GnRH may also be, due to its antigonadotropic action, involved in the regulation of follicular atresia (Gore-Langton and Armstrong, 1988).

5) Role of Inhibin, Activin, Follistatin, TGF_α and TGF_β

Inhibin and activin often exhibit opposite actions in the modulation of ovarian steroidogenesis. Inhibin augments LH-stimulated androstendione production in theca cell cultures whereas activin is inhibitory (Hasegawa et al., 1988). FSH-induced aromatase activity in granulosa cells is enhanced by bovine activin (Hutchinson et al., 1987) but is attenuated by porcine inhibin (Ying et al., 1986). Bovine inhibin has been shown to be ineffective in this regard (Hutchinson et al., 1987). Both activin and inhibin are known to reduce P secretion by granulosa cells (Hutchinson et al., 1987).

TGF_β, a member of the inhibin-activin family, augments FSH-stimulated estrogen and P production by granulosa cells, although it is ineffective in the absence of the gonadotropin (Adashi and Resnik 1986; Dodson and Schomberg 1987; Ying et al.1986; Hutchinson et al., 1987). TGF_β is known to modulate the hormonal induction of LH (Knecht et al., 1987) and EGF receptors on granulosa cells (Feng et al., 1986). Skinner et al. (1987) speculated that TGF_β by inhibiting granulosa cell growth, promotes the expression of the steroidogenic capacity of these cells, rendering them more responsive to

FSH. In thecal cells TGFβ and TGF-β_1 have been shown to inhibit hCG-stimulated P (Caubo et al., 1989) and androgens production (Caubo et al., 1989; Hernandez et al., 1990 a). This finding is in keeping with the concept that theca-derived TGFβ plays a positive paracrine role in the control of granulosa cell P and estrogen production but also functions as a negative autocrine regulator in the control of thecal androgen biosynthesis (Tsafriri et al., 1989; Hernandez et al., 1990 a). Tsafriri et al. (1989) have postulated that TGFβ, inhibin and related proteins modulate steroidogenesis at early but not late stages of follicular development of the rat ovary. The authors suggested that the steroidogenic capacity of the preovulatory Graafian follicles is stimulated consistently only by TGF$_\alpha$ but not TGFβ, inhibin, activin or follistatin during development (Tsafriri et al., 1989).

6) Role of Insulin and Insulin-Like Growth Factors (IGFs)

Insulin and IGFs stimulate ovarian steroidogenesis in an autocrine and paracrine manner. IGF-I and IGF-II receptors have been found on both granulosa and theca cells (Davoren et al., 1986; Hernandez et al., 1990 b). Although the actions of insulin, IGF-I and IGF-II are remarkably similar, IGF-I is the most and insulin is the least potent in stimulating steroidogenesis (Adashi et al., 1985; Davoren et al., 1986).

FSH and IGF-I synergistically stimulate P secretion by swine granulosa cells. This effect is not attributable to decreased catabolism to 20α-OH-P, but rather to enhanced P_5 biosynthesis (Veldhuis and Rodgers, 1986). IGF-I increases the basal (10-50 fold) production of P_5, P and 20α-OH-P by swine granulosa cell cultures in the absence of gonadotropin (Veldhuis and Furnaletto 1985). Both IGF-I and insulin enhance granulosa cell P production in response to estradiol, 8-bromo cAMP, LDL and 25-hydroxy-cholesterol (Adashi et al., 1985; Veldhuis et al.,1986b). These peptides increase the number of LDL receptors and the binding, internalization and degradation of LDL (Veldhuis et al., 1986; 1987), they also stimulate free cholesterol and cholesteryl ester accumulation (Veldhuis, et al., 1989). In addition, IGF-I enhances high density

lipoprotein (HDL)-promoted P production in swine granulosa cells in vitro (Veldhuis et al., 1989).

Recently, IGF-I and insulin have been shown to increase the abundance of mRNA encoding cytochrome $P_{-450scc}$ in porcine granulosa cells (Urban et al., 1990). Although ineffective by themselves, LDLs act synergistically with IGF-I to increase $P_{-450scc}$ mRNA and P production (Urban et al., 1990). IGF-I and E_2 synergistically enhance granulosa cell P but not $P_{-450scc}$ mRNA levels. An absence of a direct correlation between increased levels of $P_{-450scc}$ mRNA (treated with IGF-I alone or with other effectors) and P production has also been observed in rat theca interna cells (Caubo et al., 1989; Magoffin et al., 1990). It should be noted, however, that steroidogenesis in theca cells is increased by both insulin and IGF-I although the concentrations of insulin required for maximal stimulation are significantly higher then those of IGF-I (Caubo et al., 1989).

IGF-II is unable to stimulate ovarian P synthesis on its own but is capable of synergizing with FSH. It significantly enhances FSH-stimulated progestin biosynthes in granulosa cells from immature, hypophysectomized, estrogen-treated rats (Adashi et al., 1985; Davoren et al., 1986). In addition, in theca cells IGF-II does not have a significant effect on basal secretion of P, androstendione and estradiol but slightly increases that of testosterone. Treatment of the theca cells with hCG and IGF-II dose-dependently increases the secretion of all four steroids (Caubo et al., 1989).

7) Role of EGF

Depending on the species studied, EGF has variable effects on steroid hormone synthesis. Granulosa cell P production in vitro is not affected by this growth factor in swine (Caubo et al. 1989), is inhibited in bovine (Franchimont et al., 1986) and is increased in the human (Tapanainen et al., 1987) and the rat (Tippet et al., 1988). FSH-induced secretion of P_5, P and 20α-OH-P is augmented by EGF in rat granulosa cells (Jones et al., 1982). In contrast, estrogen production (Hsueh et al., 1981; Jones et al.,

1982; Caubo et al., 1989) and aromatase activity (Hsueh et al., 1981; Schomberg et al., 1983) are inhibited by EGF in human, rat and swine granulosa cells. The growth factor does not influence gonadotropin-induced P and androgen synthesis by porcine theca cells in vitro (Caubo et al., 1989) but inhibits that by the rat theca-interstitial cells (Erickson and Case, 1983).

8) Role of Cytokines

Resident ovarian (i.e. extravascular) mononuclear phagocytes (macrophages), lymphocytes and polymorphonuclear granulocytes are present in the ovary at some stages of follicular and luteal development. Of particular interest are macrophages which constitute a major cellular component of the interstitial (interfollicular) ovarian compartment and are believed to be involved in phagacytosis of atretic follicles. Macrophage-like cells, thought to be derived from granulosa cells, have been identified in atretic follicles (Gottschall and Arimura, 1990); however the precise source of macrophages in ovaries remains to be determined. It has been suggested that macrophages as well as the resident ovarian white blood cells may serve as potential in situ ovarian modulators, acting through the local secretion of regulatory cytokines (Adashi, 1989).

a. Interleukin-I (IL-I)

Interleukin-I, a polypeptide cytokine produced and secreted predominantly by activated macrophages, is known to have a variety of biological functions including its role as an immune mediator (Adashi, 1990). In the ovary, IL-I suppresses the functional and morphological luteinization of cultured murine and porcine granulosa cells (Fukoka et al., 1989; Kasson and Gorospew, 1989). IL-I_α, IL-I_β and IL-3 augment FSH-stimulated 20α-OH-P production but do not affect FSH-stimulated estrogen biosynthesis or LH receptor induction (Kasson and Gorospew, 1989). Studies by Adashi (1990) indicate that

theca-interstitial cells may also be a site of action of IL-I but not of IL-2. The activity of interleukins appears to be regulated by P. IL-I gene expression is stimulated by low but inhibited by high concentrations of P (Polan et al., 1988). IL-I has been suggested to play a role in the suppression of premature follicular luteinization.

b. Tumor Necrosis Factor α (TNFα)

Tumor necrosis factor α, another cytokine produced by the macrophages, has been immunocytochemically localized in the human, rat and bovine ovary (Terranova et al., 1991). The granulosal compartment of the antral follicle is the major site of the immunoreactive TNF_α in these species. TNF_α is capable of attenuating the differentiation of cultured rat granulosa cells (Adashi, 1990) by inhibiting FSH action at site(s) proximal to cAMP formation (Emoto and Baird, 1988). In cultured or luteinized granulosa cells, TNF_α inhibits basal and FSH-stimulated accumulation of P_5 and 20α-OH-P (Payne et al., 1990; Terranova et al., 1991).

IV. SIGNAL TRANSDUCTION IN THE OVARY

The functions of the ovary are regulated by gonadotropins and other peptide factors. Although, these factors do not cross the plasma membrane, they bind to membrane receptors and transduce the message through one of the signalling pathways.

A. System Components

1) Cellular Receptors

The ability of cells to respond to extracellular, regulatory factors (the primary messengers) depends on the presence of specific receptors. These receptors are classified either on the basis of their cellular localization (membrane, or inside the cell) or on the basis of the signal transduction mechanism to which they are physiologically connected.

According to Birnbaumer and co-workers (1985), receptors based on signal transduction mechanism are classified into four different subtypes:

1. Receptors that regulate cAMP formation;
2. Receptors coupled to the hydrolysis of membrane phosphatidylinositol phosphates that regulate the formation of diacylglycerol and inositol phosphates (which activate PKC and increase intracellular calcium levels);
3. Receptors that possess tyrosine kinase activity;
4. Receptors that are associated with ion channels and on occupancy by their specific ligands, allow flux of specific ions across the plasma membrane and thereby trigger electrophysiological response.

Receptors coupled to the adenylyl cyclase system are classified into two subtypes: R_S receptors, which increase cAMP levels by stimulating adenylyl cyclase, and R_I receptors which reduce cAMP levels by inhibiting the enzyme.

2) G-Proteins

G-proteins provide a link between the outside and interior of the cell and function as receptor-effector couplers (Spiegel et al., 1988; Birnbaumer et al., 1990; 1991) (Fig. 4). There are many primary messengers and specific receptors mediating their action but much fewer effector systems. A single effector system can be regulated by more than one G protein and a single G protein may regulate more than one effector. Several G proteins have been purified, including four G_S (stimulatory G proteins) and three G_I's (inhibitory G proteins) (Birnbaumer et al., 1991). Recently, G protein- or guanine nucleotide-regulated ion channels have been described (Birnbaumer et al., 1991).

All G proteins share certain common features. They function as receptor-effector couplers, bind guanine nucleotides with high affinity and specificity, possess intrinsic GTPase activity, serve as substrates for covalent modification by bacterial toxins and share a heterotrimeric structure (Spiegel et al., 1988). They are made up of α,β and γ subunits which dissociate into α-guanine nucleotide complexes and $\beta\gamma$ dimers during activation (Spiegel et al., 1988; Birnbaumer et al., 1990). The α subunits of G proteins bind and hydrolyze GTP. They define the receptor and effector specificity and differ between G proteins (Birnbaumer, 1990; Birnbaumer et al., 1991). Dissociation of the $\beta\gamma$ dimers from the Receptor•G_α-GTP•$\beta\gamma$-complex allows for subsequent dissociation of the receptors from the Receptor GTP-G_α such that the latter becomes free to act catalytically. By reassociation with GDP-G_α's, for which the receptors have low affinity, $\beta\gamma$ dimers guarantee reinitiation of the receptor action (Birnbaumer et al., 1991) (Fig. 4).

Although only 5 classes of G proteins have been purified, molecular cloning revealed the existence of at least 16 G_α genes and several genes coding for $\beta\gamma$'s. Not all of these genes are expressed in all cells, some are expressed only in a single cell type (Birnbaumer et al., 1991).

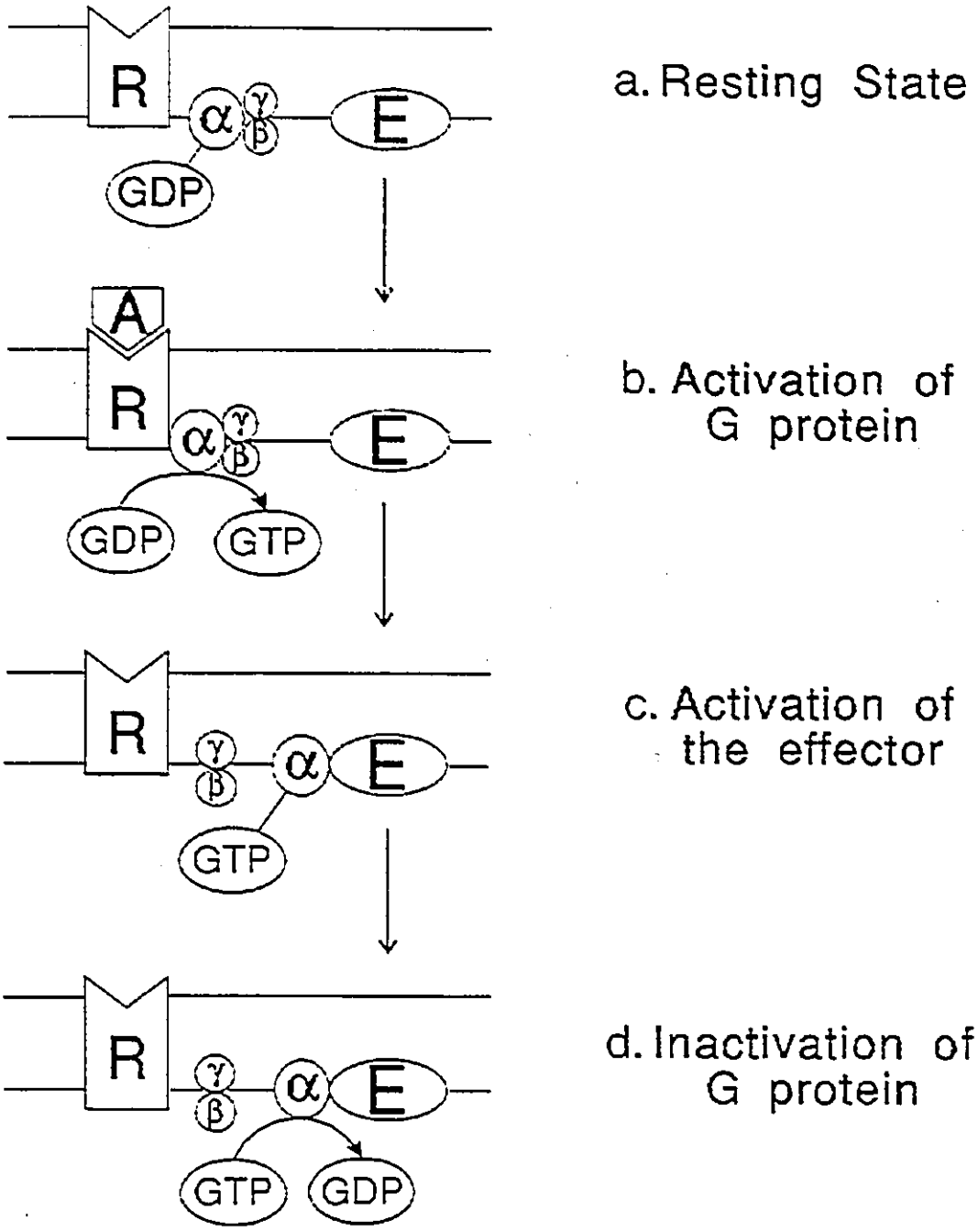

Figure 4 G-protein in agonist-induced cell signalling. (Modified after Linder and Gilman, 1992)

a.- G proteins consist of α, β and γ subunits. They are bound by GDP and have no contact with the receptor.

b.- When agonist binds to a receptor, the latter through the change in conformation binds to G protein. This interaction spurs exchange of GDP for GTP which alters the shape of α subunit and activates it.

c.- G protein dissociates and GTP-bound α subunit diffuses along the membrane, binds to an effector and activates it.

d.- After a few a seconds the α subunit hydrolyzes GTP to GDP, thereby inactivating itself and reassociates with the βγ complex.

3) Cellular Effectors

There are about as many effector systems as classes of G proteins. Ovarian effector systems most probably include:

1. adenylyl cyclase signalling system;
2. polyphosphoinositide-phospholipase C signalling system (releasing diacylglycerol and Ca^{2+});
3. phosphatidylcholine-phospholipase A_2 (releasing AA) and C or D (releasing diacylglycerol and phosphatidic acid) signalling system;
4. ionic channels - K^+, Ca^{2+} and Cl^- specific signalling system.

B. Cell Signalling Through Adenylyl Cyclase System

Gonadotropins and many other protein or peptide hormones, neurotransmitters and growth factors exert their physiological response through the adenylyl cyclase system. This system is composed of: 1) the receptor, 2) the guanine nucleotide-binding regulatory proteins (G_S and G_I) and 3) the catalytic unit (C) of the enzyme. The binding of the agonist to the receptor increases the production of cAMP, which serves as an intracellular second messenger. cAMP activates a cAMP-dependent protein kinase (PKA) which phosphorylates a variety of proteins having important physiological roles in the regulation of ovarian functions (Fig. 5).

The response of ovarian follicular cells to gonadotropic stimulation depends on the abundance and type of receptors and the nature of the G protein in the adenylyl cyclase system. FSH receptors are physically and functionally associated with stimulatory (G_S) protein (Zhang et al., 1988; 1991). Low and high affinity binding sites are present on G_S protein coupled to FSH receptor (Zhang et al., 1988; 1991). Zhang et al. (1991) suggested that, the high affinity GTP-binding sites of G_S protein are essential for the activation of adenylyl cyclase. Binding of GTP to low affinity binding sites modulates

FSH binding, but FSH receptors coupled to these sites are not involved in the regulation of the activation of the adenylyl cyclase effector system.

cAMP-dependent protein kinase (Fig. 5) is composed of two regulatory (R) and two catalytic (C) subunits that together constitute an inactive holoenzyme R_2C_2. Binding of cAMP to the regulatory subunit dimer results in the release and the concomitant activation of the catalytic subunit (Taylor, 1989; Nigg, 1990). The function of the R-subunit is to bind and inhibit the C subunit in the absence of cAMP. Two major classes of R-subunits (RI and RII) and their corresponding holoenzymes have been observed in eucaryotic cells (Taylor, 1989). In granulosa cells from preovulatory follicles, an increased number of LH receptors and enhanced responsiveness of adenylyl cyclase to FSH and LH is accompanied by a significant increase in the content of the regulatory subunit of cAMP-dependent protein kinase type II, designated as RII51 (Mr=51000). RII51 is present in both granulosa and theca cells of small antral follicles but is decreased dramatically after the LH surge (Hedin et al., 1987). RI mRNA, transiently elevated in theca cells is decreased in granulosa cells of preovulatory follicles and corpora lutea (Hedin, et al., 1987). Treatment of hypophysectomized rats with estradiol and gonadotropins increases RII51 mRNA in granulosa cells (Hedin et al., 1987). This increase is associated with an enhanced binding of [^{3}H] cAMP to RII (Richards and Rolfes, 1980). Dose-response studies show that FSH increases RII51 mRNA by three- to seven-fold and that the amount of RII51 is greatest in estradiol-primed granulosa cells (Hedin et al., 1987). It has been suggested, that an enhanced response of estradiol-primed rat granulosa cells to FSH may, at least in part, be associated with the increased ability of FSH to activate adenylyl cyclase (Richards, 1980; Richards et al., 1987). Studies using granulosa cells in serum-free cultures have demonstrated that forskolin, and 8- bromo-cAMP increase the content and synthesis of RII51 mRNA and its isoelectric variants (RII51,5 and RII52) (Richards and Hedin, 1988). The mechanisms by which FSH and estradiol regulate RII51 mRNA content and gene transcription remains to be determined.

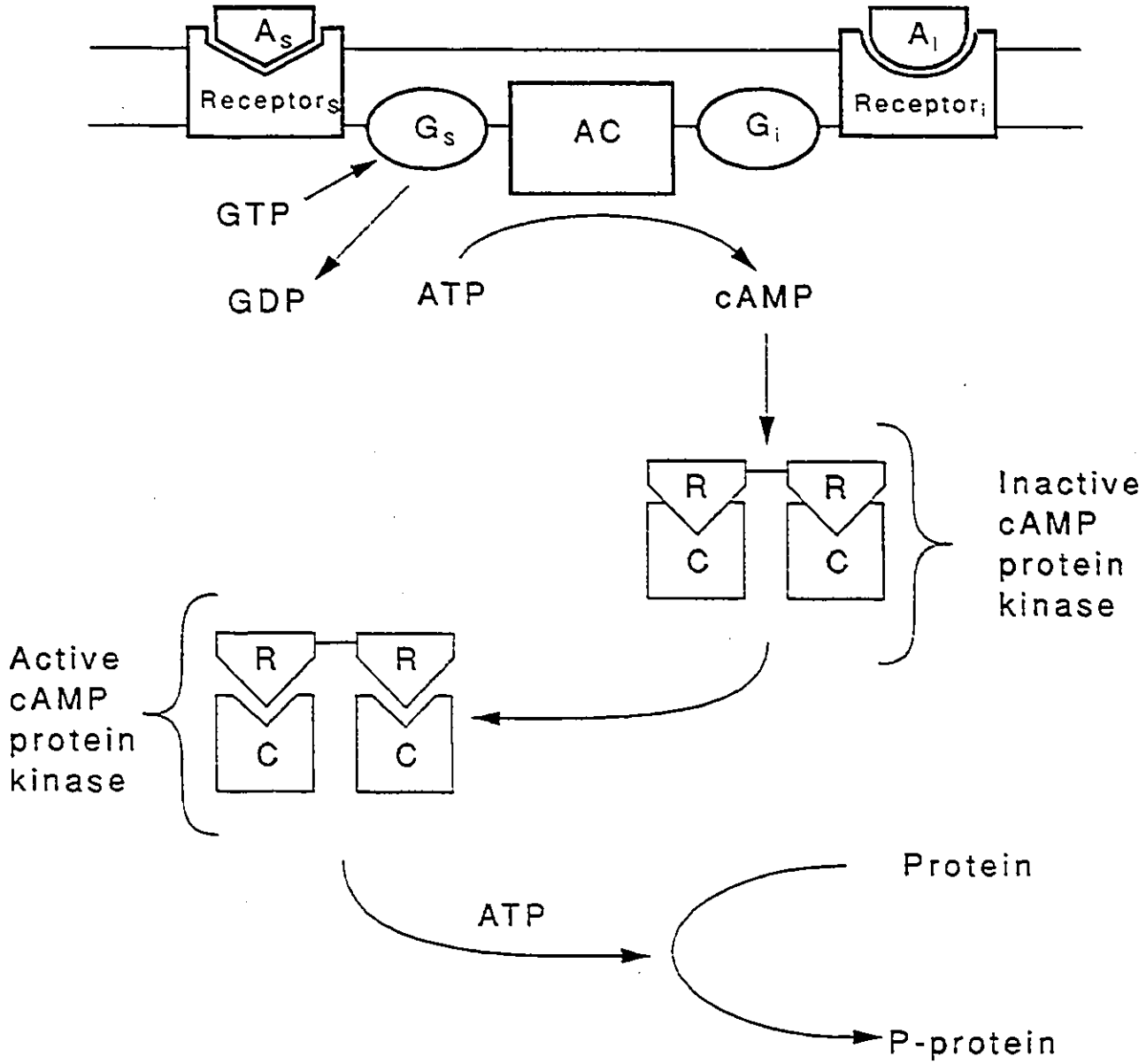

Figure 5 Signal transduction through cAMP pathway.

Stimulatory agonist (A_s) and inhibitory agonist (A_i) interact with their respective receptors (Receptor$_s$ and Receptor$_i$). The latter bind accordingly to stimulatory or inhibitory G proteins (G_s or G_i) prompting their activation and either activation or inhibition of adenylyl cyclase pathway. Stimulatory agonist via G_s leads to activation of catalytic subunit of adenylyl cycalse (AC) which activates cAMP-dependent protein kinase by dissociation of its regulatory subunits (R) from catalytic subunits (C). Activated cAMP kinase phosphorylates substrate proteins.

cAMP is believed to mediate the action of gonadotropins in the control of ovarian steroidogenesis. It regulates the differentiation of granulosa cells and the formation of gonadotropin, estradiol and LDL receptors (Amsterdam and Rotmensch, 1987; Strauss III et al., 1988). cAMP controls cellular availability of cholesterol through the stimulation of LDL and HDL delivery and metabolism (Golos and Strauss III, 1987) or de novo synthesis of the lipid and its release from the lipid droplets by increasing the activities of HMG-CoA reductase (Rodgers et al., 1987a, Golos and Strauss III, 1988) and cytosolic sterol ester hydrolase (Naghshineh et al., 1978), respectively. cAMP mediates the stimulatory action of gonadotropins on the metabolism of cholesterol to P_5 and P_5 to P. It increases the synthesis of $P_{-450_{scc}}$, ISP and NADPH:ISP reductase in granulosa cells (Trzeciak et al., 1986; Goldring et al., 1987) and theca-interstitial cells (Magoffin, 1987) and the activity of 3β-HSD in granulosa cells (McAllister and Hornsby, 1988). cAMP apparently expresses its action at the transcriptional/translational and post-translational level. The expression of genes for $P_{-450_{scc}}$ (Goldring et al., 1987; Golos et al., 1987a), ferredoxin (Golos et al., 1987a), 3β-HSD (Chedrese et al., 1990; Tremblay et al., 1991) and the LDL receptor (Golos and Strauss III, 1987) are induced by cAMP in granulosa and luteal cells. Post-translational modifications induced by cAMP include phosphorylation of enzymes involved in the regulation of cholesterol metabolism. In addition, cell shape and morphology are also mediated, at least in part, through the adenylyl cyclase messenger system (Soto et al., 1986).

C. Cell Signalling Through Inositol Lipid Metabolism

A variety of hormones, neurotransmitters and growth factors which activate cellular functions through interaction with membrane receptors, transduce their physiological signals by provoking the breakdown of inositol phospholipids in their target cells. In 1953, Hokin and Hokin discovered that acetylcholine stimulates the incorporation of ^{32}P into the phosphatidylinositol (PI) of pancreas slices. Holub (1970),

in his research on PI discovered that the lipid contains 1-stearoyl-2-arachidonyl fatty acids on the sn-glycerol backbone. The interest in PI faded for more than a decade, until Mitchell (1975) postulated that agonist-induced breakdown of membrane PI may play a role in calcium gate opening and Ca^{2+} mobilization. Mitchell's postulates were followed by the new discoveries on the role of inositol phospholipids in protein phosphorylation. Nishizuka and his co-workers subsequently discovered, that a product of phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown, 1,2-diacylglycerol, activates protein kinase C (PKC). The properties of PKC and its role in signal transduction were subsequently characterized by Nishizuka's group (Takai et al., 1979 a, b; Kishimoto et al., 1980; Mori et al., 1980; Nishizuka and Takai, 1981; Castagna et al., 1982; Nishizuka 1983; 1984; 1988; 1989). In 1983, Berridge observed that inositol 1,4,5-trisphosphate (IP_3), produced with diacylglycerol during hydrolysis of PIP_2, stimulates intercellular calcium release. The following years brought an avalanche of new research on the role of PKC, diacylglycerol, IP_3 and other products of PIP_2 breakdown in the regulation of cellular processes (Berridge 1984, 1986, 1987; Nishizuka 1984, 1988, 1989; Berridge and Irvine, 1989).

1) System Components

Inositol phospholipids comprise less than 10% of total cellular phospholipids and are very active metabolically. They have a predominantly 1-stearoyl-2-arachidonyl fatty acid composition on the sn-glycerol backbone. Small portions of PI contain one or two additional phosphates and are referred as phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2). PIP_2 is an immediate substrate of the receptor-linked enzymatic reaction which leads to production of 1-stearoyl-2-arachidonylglycerol (DG) and inositol trisphosphate (IP_3). DG and IP_3 serve as second messengers for PKC activation and Ca^{2+} mobilization, respectively (Takai et al., 1984; Nishizuka, 1986; Berridge, 1987; Berridge and Irvine, 1989).

Like the adenylyl cyclase system, the phosphatidylinositol system is composed of three elements 1) receptor, 2) guanine nucleotide-binding protein and 3) the enzyme called phospholipase C (PLC).

a. Receptors/G proteins

Examples of receptors that affect PI hydrolysis include those for vasopressin of the V_1 -type, GnRH, $PGF_{2\alpha}$ and catecholamines of the α_1 -type (Birnbaumer et al., 1985; Davis et al., 1988). Coupling of GnRH receptors to G protein has been demonstrated in bovine pituitary (Perrin et al., 1989) and luteal (Davis et al., 1991) cells. G protein-mediated stimulatory and inhibitory pathways have also been suggested to be responsible for switching on and off the hydrolysis of inositol lipids (Berridge, 1987).

The G protein involved in signal transduction through the PI system is often referred to as G_p, p standing for phospholipid (Berridge, 1987). G_p seems to be of the $\alpha\beta\gamma$ type (Birnbaumer et al., 1990). With the lack of information on its structural properties, its relationship to other G proteins, however, is uncertain. The evidence that G_p functions as a signal transducer is based primarily on the observation that the breakdown of PIP_2 to DG and IP_3 in permeabilized cells or isolated membranes can be stimulated by non-hydrolyzable guanine-nucleotide analogues (Berridge, 1987; Birnbaumer et al., 1990).

b. Phospholipase C

The agonist-induced breakdown of PIP_2, initiated by the cleavage of the phosphodiester linkage between glycerol and phosphate is catalyzed by PLC. Mammalian cells contain at least seven (Crooke and Bennett, 1989) PLC's, of which four immunologically distinct isoenzymes ie. PLC-α, PLC-β, PLC-γ, and PLC-δ are well described (Rhee et al., 1989). These four enzymes are dissimilar in their molecular size and amino acid sequences. It has been suggested that the isozymes of PLC may be

regulated differently and that different modes of regulation may account in part for the diversity of responses observed in various tissues and individual cell types to external stimuli and during development (Ryu et al., 1990, Rhee et al., 1989).

2) Phosphatidylinositol Turnover - Production of Second Messengers

The primary products of PIP_2 breakdown by PLC are DG, IP_3 and the metabolite of DG, arachidonic acid (AA), which act as the second messengers in transducing extracellular signals into intracellular events (Fig. 6).

a. IP_3/Calcium Signalling Pathway

In 1975 Mitchell postulated that PI turnover is involved in Ca^{2+}-gate opening. He also claimed that activation of Ca^{2+} mobilizing receptors stimulate PIP_2 breakdown, and hypothesized that this breakdown is essential for intracellular Ca^{2+} mobilization (Mitchell, 1975; 1982). Mitchell's hypothesis was subsequently confirmed by Nishizuka (1984 a, 1984 b) and Berridge and Irvine (1984, 1985, 1989).

Of the inositol phosphates identified in mammalian cells, only IP_3 and possibly its metabolite, inositol 1,3,4,5-tetrakisphosphate (IP_4), are believed to mobilize intracellular calcium. IP_3 regulates calcium release by inducing conformational changes in the intracellular IP_3 receptor (Berridge, 1987; Irvine, 1989; Meyer et al., 1990). These changes, perhaps in conjunction with the action of IP_4, cause the opening of Ca^{2+}-channels in both the endoplasmic reticulum and the plasma membrane (Berridge, 1990). It has been proposed that the endoplasmic reticulum, but not the mitochondria, is likely the IP_3-sensitive intracellular calcium pool. Newly discovered cellular organelles called calcisomes have been shown to contain IP_3 receptors and have been suggested as a site of IP_3 action (Volpe et al., 1988). Irvine (1989) has shown that calcisomes, possibly linked with endoplasmic reticulum, are the principal Ca^{2+}-pumping organelles. Calcisomes and at least the endoplasmic reticulum near the plasma membrane are connected by channels in

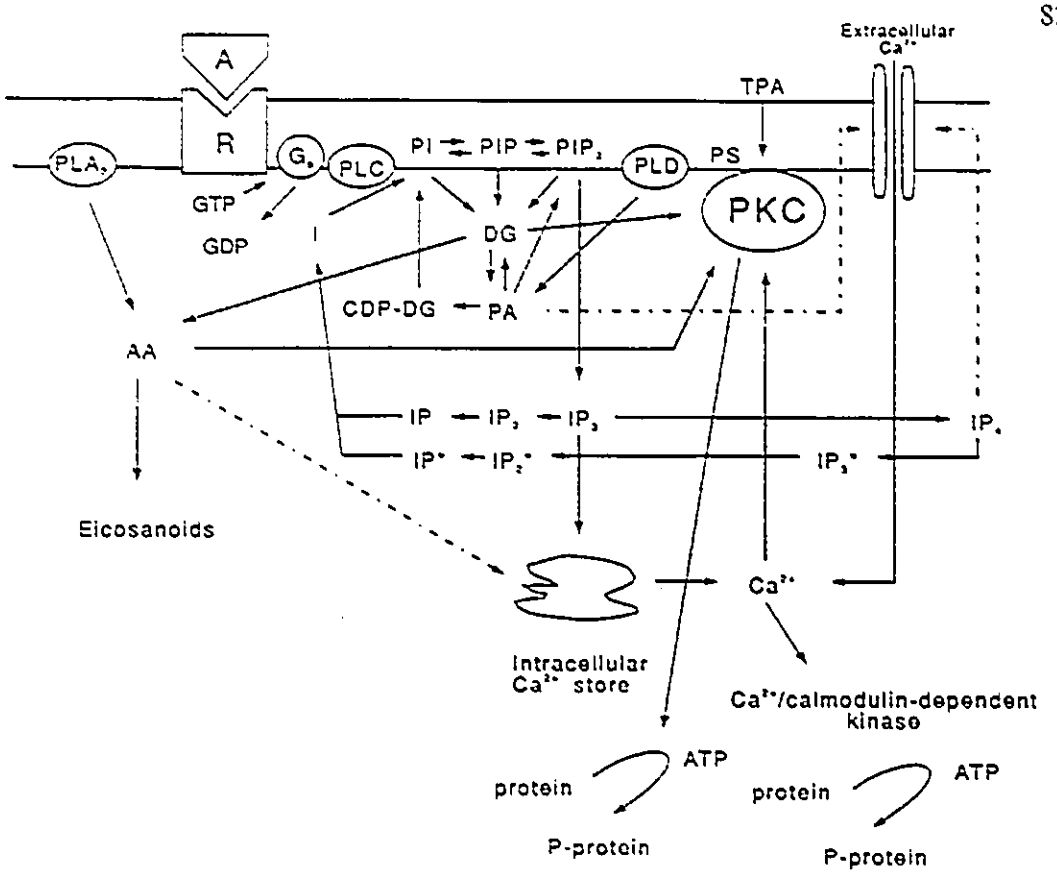

Figure 6 A model for agonist-induced phosphoinositide turnover and signal transduction.

Binding of agonist (A) to cell surface receptor (R) activates, via G protein (G_p), phospholipase C (PLC). The latter hydrolyzes phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DG) and inositol trisphosphate (IP_3). DG can also be produced from hydrolysis of phosphatidylinositol (PI) and phosphatidylinositol monophosphate (PIP) or from phosphatidic acid (PA) released during hydrolysis of other membrane phospholipids by phospholipase D (PLD). DG activates protein kinase C (PKC) which phosphorylates and activates proteins; IP_3 mobilizes Ca^{2+} from intracellular stores. IP_3 is metabolized to inositol tetrakisphosphate (IP_4), believed to induce Ca^{2+} influx via Ca^{2+} channels, and then to an inactive IP_3 (IP_3^*) isoform which is further metabolized to inositol biphosphate (IP_2^*) and inositol phosphate (IP^*) isoforms and free inositol (I). An alternative pathway of IP_3 metabolism also leads to subsequent dephosphorylation of the lipid to I. Increased Ca^{2+} activates Ca^{2+}/calmodulin - dependent protein kinase. It may also activate PKC. DG is rapidly metabolized to PA (thought to stimulate IP_3 formation and/or Ca^{2+} influx) and then to cytosine diphosphate-DG (CDP-DG) which reacts with I to reform PI. Alternatively DG can be converted to arachidonic acid (AA) (also metabolized from membrane phospholipids eg. phosphatidylcholine, during activation of phospholipase A_2 (PLA_2)). AA activates PKC and is believed to mobilize Ca^{2+} from intracellular stores. Tumor promoting phorbol ester (TPA) also activates PKC and is involved in the regulation of intracellular Ca^{2+} mobilization.

a manner analogous to gap junctions. These channels are obligatorily controlled by IP_4. According to Irvine (1989), during activation of inositide-linked receptors, both second messengers, IP_3 and IP_4, are involved in the control of intracellular Ca^{2+} concentration (Fig. 6). Release of calcium from intracellular stores induced by the external stimulus and mediated by IP_3 and IP_4 is quantal (oscillatory) in nature and occurs during the first 1-4 sec. of stimulation (Berridge, 1990). Ca^{2+} is then removed from cytosol by Ca^{2+} pump and retaken into intracellular stores. Reloading of the internal Ca^{2+} stores requires termination of the stimulus, since the membranes of the organelles remain permeable to Ca^{2+} when cytosolic levels of IP_3 are high, as occurs during signal induced PIP_2.

Intracellular Ca^{2+} is involved in the regulation of a variety of physiological reactions. It is needed for activation of Ca^{2+}/calmodulin-dependent protein kinase (Means and Dedman, 1980; Manalan and Klee, 1984) and Ca^{2+}/phospholipid-dependent PKC (Nishizuka 1984 a,b; 1988). Ca^{2+} has been suggested to act either on its own, as a second messenger or synergistically with other signalling systems (e.g. PKC) (Nishizuka 1984 a,b; Rasmussen et al., 1984; Berridge 1986; 1987).

b. Protein Kinase C Signalling Pathway

DG is produced during agonist-induced breakdown of PIP_2, however, it can also be formed from hydrolysis of PIP and PI. It is present transiently in the cell membrane and is rapidly metabolized to phosphatidic acid (PA) (Nishizuka et al., 1984, a,b; Takai et al., 1984; Berridge, 1987). PA can either react with CTP to produce CDP-diacylglycerol (Hokin, 1968) or can be acted upon by DG lipase to form AA (Murayama and Ui, 1985; Leung and Wang, 1989). CDP-diacylglycerol reacts with inositol to reform phosphatidylinositol, and AA is metabolized to eicoisanoids (Bell et al., 1979) (Fig. 6).

The primary function of diacylglycerol is to activate PKC (Nishizuka, 1984 a,b) (Fig. 7). However, diacylglycerol and PA have also been suggested to play a role in calcium mobilization (Kroll et al., 1989). Diacylglycerol might be acting as a membrane

perturber (Allan et al., 1975, 1976) and PA as a calcium ionophore (Gerrard et al., 1978; Salmon, 1980; Putney et al., 1980), thus enabling Ca^{2+} flux from extracellular space. In addition, PA might stimulate the formation of IP_3 (Moolenaar et al., 1986). Recent data implicate an involvement of diacylglycerol in the PKC-dependent regulation of calcium-activated K^+ channels and/or Na^+/H^+ exchanger, which modulates intracellular pH (Berridge 1986; Li et al., 1991).

Information on the structure-activity relationship of diacylglycerols with PKC activity are incomplete. However, the active diacylglycerols appear to contain at least one unsaturated fatty acid and are independent of the length of the other fatty acyl moiety (Mori et al., 1982; Nishizuka 1984, a,b).

Although the production of diacylglycerol is known to result from the hydrolysis of PIP_2 in response to a variety of cellular regulators, recent data suggest that it is also released during signal-induced breakdown of membrane phosphatidylcholine. (Exton, 1990). Hydrolysis of PIP_2 is primarily responsible for the early increase in diacylglycerol, while phosphatidylcholine breakdown is believed to be important for a sustained generation of diacylglycerols and thus the maintenance of PKC activation (Matozaki and Williams, 1989; Exton, 1990).

c. Arachidonic Acid Signalling Pathway

AA is a common substituent of DG. The acid is released from the 2-acyl position of membrane phospholipids by various phospholipases including PLC, phopholipase A_2 (PLA_2) and/or phospholipase D (PLD). During PLC-induced breakdown of membrane PIP_2 AA is produced from PA via monoacylglycerol (Nishizuka, 1984; Berridge, 1987) but can also be released after translocation of diacylglycerol kinase to membrane-bound compartments and activation of PLA_2 (Kolesnik and Paley, 1987). PLA_2 and PLD can directly act on membrane phospholipids (Nishizuka et al., 1984). These enzymes have

been shown to metabolize phophatidylcholine (PC) to PA, which may be converted to DG and then to AA (Kolesnik and Paley, 1987; Kroll et al., 1989).

There is a growing body of evidence that AA and related fatty acids play an important role in agonist-induced signal transduction. Several investigators reported that AA activates PKC (McPhail et al., 19 84; Naor et al., 1988; Seifert et al., 1988) and influences intracellular calcium mobilization (Leung and Wang, 1989) (Fig. 6). However, as shown in hen granulosa cells, it can also activate cellular processes independently of PKC (Johnson and Tilly, 1990).

GnRH and $PGF_{2\alpha}$ seem to stimulate the production of AA in rat granulosa (Minegishi and Leung, 1985; Kawai and Clark, 1986; Leung and Wang, 1989) and luteal (Watanabe et al., 1990) cells. Leung and Wang, (1989), have suggested that AA mediates predominantly the stimulatory but not the inhibitory aspects of GnRH action in the regulation of ovarian steroidogenesis .

3) Protein Kinase C (PKC)

PKC has been implicated in the regulation of many cellular processes including growth, differentiation, sensory transduction, hormone and neurotransmitter release, gene expression and cellular metabolism (Nishizuka et al.,1984; Berridge 1986, 1987). PKC is a family of closely related multifunctional protein kinases, known to phosphorylate a range of cellular proteins (Carpenter et al., 1987; Nishizuka, 1988, 1989; Soderling, 1990). The threonine-serine specific kinase was first purified from rat brain cytosol as a protein with an apparent molecular weight of 80 kD. It is sensitive to three principal regulatory factors: calcium ions, phosphatidylserine and diacylglycerols (Inoue, et al., 1977; Kishimoto et al., 1980; Kikkawa et al., 1982). PKC owes its current prominence to the findings by Nishizuka and co-workers, that diacylglycerol is likely to be the second messenger regulating the activity of PKC under physiological conditions (Nishizuka et al., 1984) and that tumor promoting phorbol ester, 12-O-tetradecanoylphorbol 13-acetate

(TPA), can activate PKC in a fashion similar to diacylglycerol (Castagna et al., 1982; Kikkawa et al., 1984; Nishizuka et al., 1984).

PKC is present in all mammalian cells thus far tested (Nishizuka et al., 1984; Nishizuka, 1988). In many of them the activity of PKC exceeds that of protein kinase A (Kikkawa et al., 1982). In addition, different isozymes of PKC have been shown to be present in different tissues and subcellular locales (Ohno et al., 1987; Yoshida et al., 1988).

a. Properties of PKC

PKC is a single polypeptide chain with two functionally different domains: a hydrophobic domain that binds to membranes and a hydrophilic domain that carries the catalytically active center (Carpenter et al., 1987). The subspecies of PKC sequenced contain the catalytic domain in the highly conserved C-terminal third of the polypeptide and the regulatory domain with recognition sites for phosphatidylserine, Ca^{2+} and diacylglycerol or phorbol ester in the amino-N-terminal (Nishizuka, 1988, 1989; Parker et al., 1986, 1989).

Under resting conditions in most tissues PKC is present in an inactive form, presumably in soluble cytosol or loosely bound to cellular membranes. It is activated by tight association with membranes during activation of PIP_2 breakdown by specific agonists (Takai et al., 1979 a; Kishimoto et al., 1980) (Fig. 7). The majority of PKC isoforms require Ca^{2+} and phospholipid for their activation. Kinetic analysis indicates that only a small amount of diacylglycerol is needed to markedly increase the affinity of the enzyme for Ca^{2+} and phospholipid thus rendering PKC fully active without a net increase in calcium concentrations (Kishimoto et al., 1980; Kaibuchi et al., 1981). This unique effect was believed to be specific for diacylglycerol, since other neutral lipids (like triacylglycerol and monoacylglycerol) appeared to be inactive (Nishizuka et al., 1984). However, unsaturated fatty acids (eg. oleic acid or AA) have recently been shown to

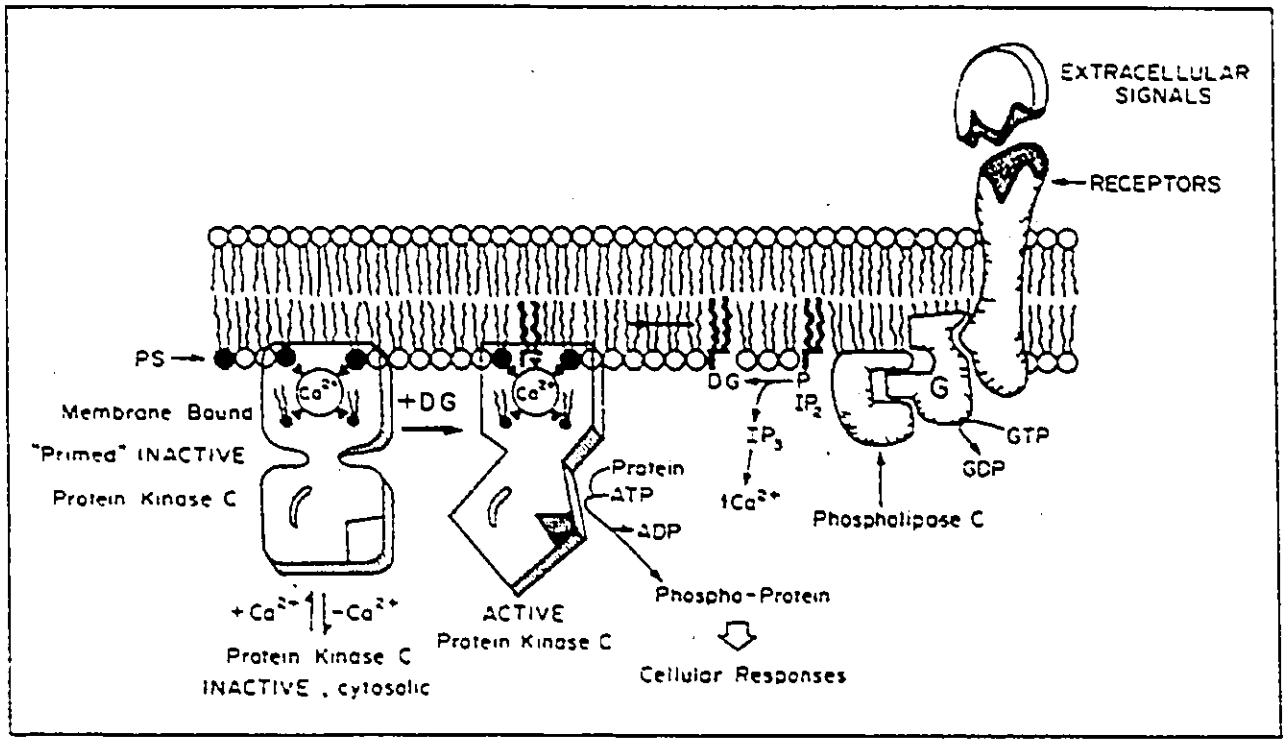

Figure 7 A model for mechanism of Protein Kinase C activation by diacylglycerol second messengers (After Bell, 1986).

Protein kinase C and Ca^{2+} interact with the phospholipid head groups of membrane. Four molecules of phosphatidylserine (PS) form a structure on the surface of the membrane, and bind Ca^{2+} through their carboxyl groups. Protein kinase C binds to this complex of PS and Ca^{2+}; in this state, the enzyme is "primed" but inactive. Diacylglycerol (DG) is produced in response to extracellular signals (depicted hypothetically as a sequence of events involving G protein - dependent activation of phospholipase C) and moves rapidly in the lateral plane of the membrane. It interacts through three points of contact with the protein kinase C-4PS-Ca^{2+} complex. Possibly a direct bond forms between DG and Ca^{2+}. Formation of the three bonds between DG and the complex-one to Ca^{2+} and two to protein kinase C-may affect a conformational change that activates the enzyme.

stimulate PKC activity in the presence or absence of Ca^{2+} or phospholipid (Naor et al., 1988; Seifert et al., 1988). Of all membrane phospholipids, only phosphatidylserine activates PKC at low concentrations of Ca^{2+} (Takai et al., 1979 b). Other phospholipids show variable effects in the activation of the enzyme (Takai et al., 1979 b).

PKC phosphorylates a variety of proteins, including PLC. The enzyme has also been shown to undergo autophosphorylation on multiple serine-threonine residues (Soderling, 1990). Recently, pseudosubstrate "autoinhibitory" domains have been identified in all subspecies of PKC. It has been suggested that the "autoinhibitory" domain which is located within the enzyme interacts with the catalytic site, thus blocking binding of substrates. Binding of the allosteric activators induce conformational changes in the autoinhibitory domain and disrupts its interaction with the catalytic domain, thereby activating the enzyme to phosphorylate exogenous substrates (Soderling, 1990).

b. Activation by Synthetic Diacylglycerols

Synthetic diacylglycerols have been used to activate PKC in membrane preparations and in intact cells in vitro (Mori et al., 1982). Particularly, 1-oleoyl-2-acetyl-glycerol (OAG) (Fig. 8) in dilute dimethyl sulfoxide (DMSO) readily intercalates into the membrane phospholipid bilayer solution and activates PKC directly without interaction with cell surface receptors (Nishizuka, 1984, a,b). During PKC activation, the synthetic diacylglycerol is rapidly converted in situ to the corresponding phosphatidic acid, 1-oleoyl-2-acetyl-3-phosphoryl-glycerol, presumably by the action of diacylglycerol kinase (Nishizuka, 1984, a,b). Other permeable diacylglycerols, like diolein and 1,2-dioctanoylglycerol (DiC_8) can also activate PKC in vitro. However, unlike permeable diacylglycerols (OAG and DiC_8), diolein cannot enter the cell even when dissolved in the vehicle (DMSO) but can activate PKC in cell-free systems.

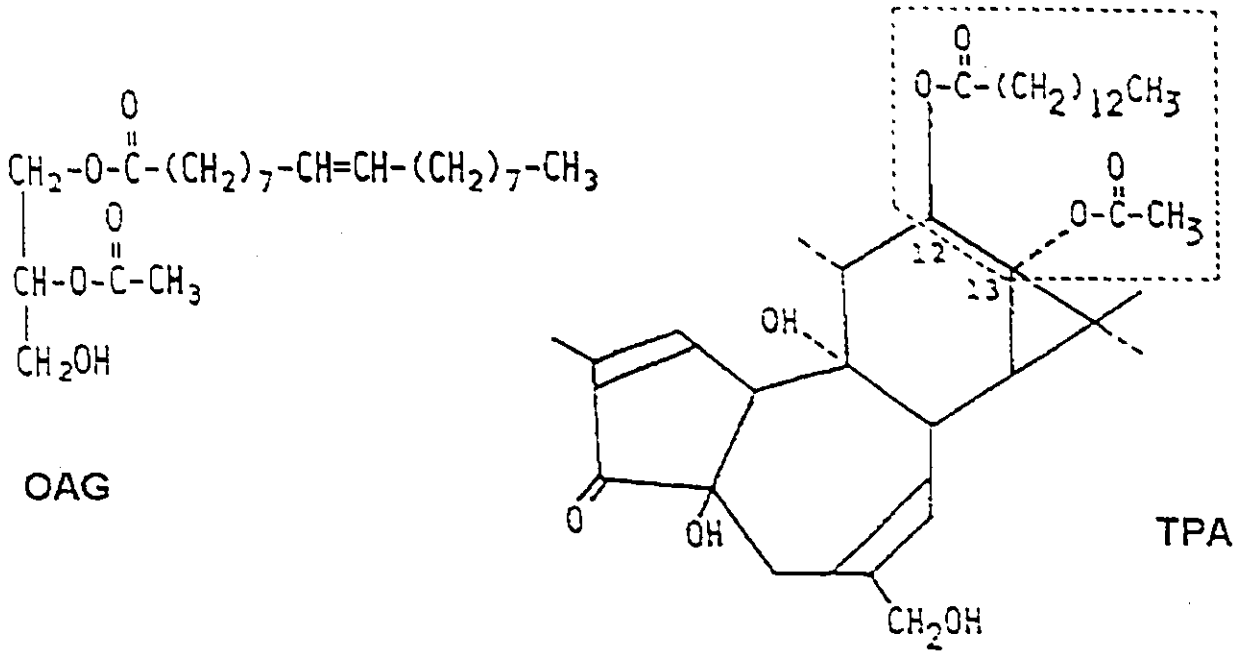

Figure 8 Structure of 1-oleoyl-2-acetylglycerol (OAG) and 12-0-tetradecanoylphorbol-13-acetate (TPA). TPA contains a diacylglycerol-like structure in its molecule (shown in the dotted square).

c. Activation by Tumor Promoting Phorbol Esters

TPA has been shown to activate PKC in a fashion similar to diacylglycerol (Castagna, 1982; Nishizuka, 1984). Since TPA receptors copurify with PKC (Niedel et al., 1983; Kikkawa et al., 1984; Parker et al., 1986, 1989), it has been assumed that phorbol ester acts by mimicking the action of OAG through the activation of the enzyme. Evidence from Nishizuka's laboratory established that TPA indeed activates PKC both in vivo and in vitro and PKC serves as a receptor protein for tumor promoting phorbol esters.

TPA has a diacylglycerol-like structure in its molecule (Fig. 8) and like DG activates PKC without net increase of Ca^{2+} or phospholipid (Castagna et al., 1982), although the latter are obligatory for activation of most of the PKC isoforms. TPA is active in extremely low concentrations and does not need DMSO (as a vehicle) to be effective. Scatchard analysis of phorbol ester binding to PKC has shown that approximately one molecule of TPA is bound to one molecule of the enzyme (Kikkawa et al., 1984). Experiments also indicate that the potent tumor promoters: TPA, phorbol-12,13-dibutyrate and phorbol-12,13-didecanoate compete equally for binding to the enzyme, while phorbol derivatives with no tumor-promoting activity can neither activate PKC, nor compete with tumor promoters for PKC binding (Nishizuka, 1984, a). TPA is not readily metabolized and remains in cellular membranes for a prolonged period. TPA has been shown to intercalate with cellular membranes and to cause insertion of PKC into the hydrocarbon region of the membrane (Bazzi and Nelsestuen, 1988). Prolonged incubation with TPA causes down-regulation of PKC. TPA has been reported to decrease intracellular Ca^{2+} levels by stimulating Ca^{2+} efflux (Rickard and Shaterline, 1985; Pollock et al., 1987; Furukawa et al., 1989), inhibiting Ca^{2+} influx (Sagi-Eisenberg et al., 1985, He et al., 1988) and/or inhibiting mobilization of Ca^{2+} from intracellular stores (Owen, 1988; He et al., 1988). Whether TPA affects other cellular functions remains to be determined.

d. Activation By Other Factors

Recent studies have shown that factors other than DG , released during PIP_2 breakdown, are also involved in the activation of PKC . It has been proposed that diacylglycerols accumulated during signal-promoted breakdown of phosphatidylcholine (PC) may also activate PKC (Exton, 1990). These diacylglycerols have higher content of palmitic, oleic and linoleic acids than expected from the breakdown of PIP_2, which is enriched in stearic and arachidonic acids (Boeckino et al., 1985). Activation of PKC due to PC hydrolysis seems to last longer than that due to PIP_2 breakdown (Wright et al., 1988; Pessin and Raben, 1989). The PC signalling pathway may be involved in the regulation of transcription and other events related to prolonged stimulation, including cell growth and differentiation, control of ion channels activity and alterations in receptor function (Exton, 1990).

In addition, PKC can also be activated by high concentrations of calcium in the absence of other second messengers (Nishizuka et al., 1984). A large increase in Ca^{2+} has been shown to activate PKC and induce non-specific degradation of phospholipid to produce diacylglycerol (Nishizuka et al., 1984). Increased levels of intracellular Ca^{2+} have also been suggested to stimulate AA production in rat granulosa cells (Kawai and Clark, 1986; Wang and Leung, 1988).

AA, released during PIP_2 breakdown, is believed to activate PKC in a variety of mammalian tissues (McPhail, et al., 1984; Naor et al., 1988; Seifert et al., 1988) including the ovary (Wang and Leung, 1988, 1989).

e. Endogenous and Synthetic Inhibitors

The metabolites of the membrane sphingolipids: sphingosine, N,N-Dimethylsphingosine (DMS) and lysosphingosine have been suggested to be endogenous inhibitors of PKC, thus modulating the stimulatory effect of 1,2-diacylglycerol

on the enzyme (Hannun and Bell, 1988; Oishi et al., 1988). Lysosphingolipids however, have a dual effect on PKC: stimulatory at low concentrations and inhibitory at higher ones (Oishi et al., 1988)

A number of inhibitors of PKC have been synthesized. They include dibucaine (Mori et al., 1980), chlorpromazine (Mori et al., 1980; Wise et al., 1982), Verapamil (Mori et al., 1980), amiloride (Besterman et al., 1985), staurosporine (Tamaoki, 1986), and 1-(5-isoquinolinesulfonyl)-3-methylpiperazine (H_7) (Hidaka et al., 1984). Aminoacridines, acridine orange, acridine yellow G and related compounds have also been shown to inhibit PKC activity and phorbol ester dibutyrate binding (Hannun and Bell, 1988). With the exception of H_7, staurosporine and sphingoid bases, little data is available regarding the mechanisms by which these inhibitors affect PKC activity. H_7 and staurosporine appear to act by competitive inhibition of ATP binding and sphingoid bases by direct inhibition of PKC activity (Sender Baum and Ahren, 1988; Hakomori, 1990). Other PKC inhibitors seem to act in a non-specific manner, perturbing the lipid bilayer and consequently inhibiting the activity of the enzyme (Hannun and Bell, 1988).

f. Multiple Subspecies of PKC

Two major categories of PKC, each with many subspecies, have been cloned and described (Carpenter et al., 1987; Ohno et al., 1988 a,b; Nishizuka, 1988; Soderling, 1990). The first category, grouping α, β_I, β_{II} and γ subspecies, requires calcium, phospholipid (phosphotidylserine) and diacylglycerol for activation. The second category, grouping δ, ε, and ε^1 subspecies, has no requirement for these co-factors (Ohno et al., 1988, a,b). All subspecies of PKC contain stretches of conserved and variable regions. Four conserved (C_1-C_4) and five variable (V_1-V_5) regions have been characterized in isoenzymes of PKC. C_3 and C_4 correspond to the protein kinase domain and have greatest homology with the cAMP - and cyclic GMP - dependent protein kinases (Carpenter et al., 1987). Ca^{2+}, diacylglycerol and phospholipid-dependent category of

PKC subspecies contain a conserved tandem repeat of cysteine-rich sequence (C_1) as well as a second highly conserved (C_2) region in the N terminal half of the polypeptide (Parker, et al., 1986, 1989). It has been determined that the regulatory domain of the first category contains both C_1 and C_2 sequences. However, the second category of PKC differs from the first one in that its subspecies have only the first cysteine-rich sequence of the tandem (Ohno, 1988, a,b). PKC deletion mutants expressed in COS-7 cells verify the importance of region C_1 and C_2 for the regulatory properties. Deletions within C_1 result in loss of phorbol ester binding, whereas deletions in C_2 appear to affect the affinity of PKC for Ca^{2+} (Kaibuchi et al., 1989). In addition, the diversities between members of the PKC family are due to differences in the variable regions. In the carboxyl-terminal catalytic domain there are two variable-region stretches, V_4 and V_5, which have been suggested to define substrate specificities (Carpenter, et al., 1988).

Different subspecies of PKC have been shown to be distinctively distributed and expressed in different tissues and different locales (Yoshida et al., 1988; Hidaka et al., 1988). Some of the isoforms are found ubiquitously among various tissues, while other predominate in very specific intracellular localizations. Besides plasma membranes and cytosol, isozymes of PKC have also been found in the nucleus (Masmoudi et al., 1989), mitochondria (Noland and Dimino, 1986) Golgi apparatus (Saito et al., 1989) and in cytoskeletal elements (Zalewski et al., 1988; Mochly-Rosen et al., 1991).

4) The Role of PKC in the Regulation of Ovarian Steroidogenesis

PKC is present in steroidogenic tissues, including the adrenal cortex (Vilgrain et al., 1984), the testis (Kimura et al., 1984; Nikula et al., 1987) and the ovary (Davis and Clark, 1983; Noland and Dimino, 1986). The presence of the enzyme has been demonstrated in bovine (Davis and Clark, 1983), human (Clark et al., 1983) and swine (Wheeler and Veldhuis, 1987) luteal tissue and in porcine (Noland and Dimino, 1986; Wheeler and Veldhuis, 1989) granulosa cells. PKC is localized mainly in ovarian cytosol

and in the particulate fractions of mitochondria and microsomes. The cytosolic fraction of medium size follicles of porcine ovaries has been shown to contain 85-91% of total PKC activity and there was no significant difference in the specific activity of cytosolic PKC in follicles of different sizes (Noland and Dimino, 1986). However, there seems to be a species-dependent variation in the activity of the enzyme. Cytosol prepared from the ovaries of immature rats treated with PMSG (Clark et al., 1980) exhibited less PKC activity (Davis and Clark, 1982) than bovine or human cytosol (Davis and Clark, 1983; Clark et al., 1983).

Although PKC is present in ovarian granulosa and luteal cells, results from various laboratories concerning its precise physiologic role are equivocal. The changes in steroid hormone production in granulosa cells seem to depend on the incubation conditions (concentration of PKC activators, duration of cell culture) and the species studied. TPA, commonly used in the granulosa cell cultures as a PKC activator, stimulated basal P_4 (Welsh et al., 1984) and P production by rat granulosa (Kawai and Clark, 1985; 1986; Shinohara et al., 1986; Wang and Leung, 1987) and luteal (Brunswig et al., 1986) cells in short (up to 6 h) incubation periods. However, in swine granulosa cells cultured for 48 h in the presence of this phorbol ester, all but very low concentrations of TPA (<1ng/ml; Hylka et al., 1989) had an inhibitory effect on the progestin production (Veldhuis and Demers, 1986; Hylka et al., 1989). Treatment of differentiated rat granulosa cells with synthetic diacyglycerol also resulted in increased steroidogenesis (Shinohara et al. 1986). However, P production by swine granulosa cells was inhibited during 15 min. incubation with diacyglycerol (Sadighian et al., 1989).

PKC has been shown to play an important role in the regulation of gonadotropin-stimulated steroidogenesis (Shinohara et al., 1985; Veldhuis and Demers, 1986; Wheeler and Veldhuis, 1988, 1989; Leung and Wang, 1989). In addition, there have been reports on the stimulatory effect of LH on de novo biosynthesis (Farese, 1983; Davis et al., 1984) and accumulation (Davis et al., 1987) of inositol lipids in ovarian tissues. LH increased

IP_3, diacylglycerol and Ca^{2+} levels in isolated bovine luteal (Davis et al., 1987) and rat granulosa cells (Davis et al., 1986). This effect of LH indicates the possibility that the gonadotropin may exert its action, at least partially, through the activation of the PKC pathway. A direct action of FSH on inositol lipid metabolism and PKC activation has not been observed. GnRH and GnRH-like hormone induced rapid turnover of PI, and formation of inositol phosphates and diacylglycerol in rat granulosa cells (Leung et al., 1983; Minegishi and Leung, 1985; Ma and Leung, 1985; Davis et al., 1986; Leung et al., 1986). It has been suggested that inositol phosphates (eg. IP_3) and diacylglycerol mediate GnRH action through activation of PKC and Ca^{2+} signalling (Nishizuka, 1984; Berridge, 1987). Like GnRH, $PGF_{2\alpha}$ also caused the hydrolysis of polyphosphoinositides and the accumulation of inositol-1-phosphate, inositol-1, 4-biphosphate and inositol 1,4, 5-trisphosphate (Leung et al., 1986; Davis et al., 1987) and may possibly activate PKC.

Agonist-activated PKC influences mitochondrial access of cholesterol by catalyzing the phosphorylation of sterol carrier protein 2 (SCP_2)(Steinschneider et al., 1989) and affects the activities of steroidogenic enzymes (Welsh et al., 1984; Trzeciak et al., 1987; Chedrese et al., 1990; Tremblay et al., 1991). Studies on FSH- or LH/hCG-stimulated rat (Welsh et al., 1984; Kawai and Clark, 1985; 1986; Trzeciak et al., 1987; Wang and Leung, 1987; Komorowski and Tsang, 1990), swine (Veldhuis and Demers, 1986; Hylka et al., 1989) and avian (Tilly and Johnson, 1988; Asem and Tsang, 1989) granulosa cells incubated for various durations and with various concentrations of TPA indicated that, regardless of the incubation conditions and animal model, TPA had a predominantly inhibitory action on gonadotropin-induced P and P_5 secretion. In addition, phorbol ester significantly inhibited P production in highly steroidogenic oncogene-transformed granulosa cells stimulated by 8-Bromo-cAMP, forskolin and cholera toxin (Suh and Amsterdam, 1990). TPA suppressed FSH-stimulated synthesis of the cholesterol side-chain cleavage enzyme complex and decreased cAMP accumulation and P formation in rat ovarian granulosa cells (Trzeciak et al., 1987). Dibutyryl cAMP ($(Bu)_2$cAMP)-

induced increase in levels of mRNA encoding the precursor form of $P_{-450scc}$ were also inhibited by TPA (Trzeciak et al., 1987). In addition, 3β-HSD mRNA abundance stimulated by LH, hCG as well as cholera toxin, forskolin and cAMP analogs was significantly reduced by the phorbol ester in cultured porcine granulosa cells (Chedrese et al., 1990 b). LH-induced 3β-HSD activity in rat granulosa cells was similarly attenuated by TPA (Kawai and Clark, 1986). This is contrary to the earlier studies which indicated that the activity of the enzyme in rat granulosa cells treated with FSH was slightly increased by TPA in vitro (Welsh et al., 1984). The activity of 20α-HSD was significantly stimulated by phorbol ester in rat granulosa cells in the presence or absence of gonadotropins during short and long incubations (Welsh et al., 1984; Kawai and Clark, 1986).

The effect of exogenous diacylglycerols on gonadotropin-stimulated progestin biosynthesis has not been investigated thoroughly, since it is commonly assumed that these PKC activators and TPA evoke similar physiological responses. Like TPA, DiC_8 attenuated FSH-stimulated P secretion in swine granulosa cells in vitro (Veldhuis and Demers, 1986). In addition, LH-stimulated P secretion by hen granulosa cells was also inhibited by another synthetic diacylglycerol - OAG (Tilly and Johnson, 1988).

GnRH, is believed to regulate steroidogenesis through the activation of PKC and to affect progestin secretion in a way resembling the action of TPA. Treatment of granulosa cells with GnRH increased basal production of P during short term incubation (up to 5 h) (Wang and Leung, 1987) but attenuated gonadotropin-stimulated biosynthesis of this steroid (Knecht et al., 1985; Shinohara et al., 1985; Kawai and Clark, 1986). The PKC inhibitor H_7, abolished the effect of both TPA and GnRH on basal secretion of steroids (Wang and Leung, 1987). GnRH has been shown to cause a rapid and transient increase in intracellular calcium in granulosa cells (Wang et al., 1989).

Like GnRH, $PGF_{2\alpha}$ produced by ovarian cells is believed to exert its steroidogenic action through the phosphatidylinositol signalling pathway (Leung, 1985; Lahav et al.,

1988, Watanabe et al., 1990). The inhibitory effect of $PGF_{2\alpha}$ on gonadotropin-stimulated secretion of P can be mimicked by tumor promoting phorbol ester. TPA significantly reduced LH-stimulated cAMP and P secretion in luteal cells (Sender Baum and Rosberg, 1987). It also inhibited steroid synthesis induced by 8-bromo-cAMP and $(Bu)_2$cAMP (Sender Baum and Rosberg, 1987). The levels of $PGF_{2\alpha}$ known to inhibit P secretion in rat luteal cells were similar to those which increased labelling of phosphatidic acid and phosphatidylcholine with ^{32}P (Raymond et al., 1983).

5) The Role of Calcium in the Regulation of Ovarian Steroidogenesis

Ca^{2+} appears to be vital for ovarian steroid synthesis. Omission of Ca^{2+} from granulosa cell incubations markedly reduced P production (Veldhuis and Klase, 1982; Carnegie and Tsang, 1983; Tsang and Carnegie, 1983). Moreover, addition of lanthanum (a blocker of cellular Ca^{2+} intake) or EGTA (calcium chelator) to granulosa cells medium resulted in significant decrease in basal and FSH-stimulated cAMP and P production during short term incubations (up to 6 h) (Carnegie and Tsang, 1983; Tsang and Carnegie, 1983). Removal of Ca^{2+} from the extracellular medium also attenuated the steroidogenic response induced by LH, 8-bromo cAMP, $PGF_{2\alpha}$ and PGE_2 in swine and bovine luteal cells (Alila et al., 1989).

Calcium ionophores have been shown to increase basal P_5, P and 20α-OH-P secretion (Tsang et al., 1988; Tsang et al., 1989) but to inhibit gonadotropin-stimulated P synthesis (Leung et al., 1988) in rat and swine (Veldhuis et al., 1984) granulosa cells in vitro. In the gonadotropic control of ovarian steroidogenesis Ca^{2+} is required for the regulation of cAMP level(s) and some step(s) on steroidogenic pathway distal to cAMP cascade (Veldhuis and Klase, 1982 a, b; Carnegie and Tsang, 1983, 1984; Asem and Hertelendy, 1986). Although, the site of action of Ca^{2+} in rat granulosa cells is not fully known, the ion appears to be particularly involved in the regulation of the step on steroidogenic pathway catalyzed by 3β-HSD (Tsang et al., 1989).

GnRH, LH and FSH have been shown to increase the intracellular Ca^{2+} concentration in steroidogenic cells. GnRH, an activator of the PI signalling pathway (Ma and Leung, 1985; Davis et al., 1986) acutely elevated intracellular Ca^{2+} levels. The accumulation of inositol phosphates correlated well with the time course of GnRH-stimulated Ca^{2+} changes in individual rat granulosa cells (Wang et al., 1989). Moreover, GnRH acutely increased basal P production but inhibited the steroidogenic response to gonadotropin, cAMP and cholera toxin stimulation. The effect of GnRH could be mimicked by A23187 and TPA. These latter agonists also acutely stimulated basal P production but attenuated steroidogenesis in the presence of gonadotropin (Leung et al., 1988; Leung and Wang, 1989).

FSH and LH have been shown to evoke an increase in intracellular Ca^{2+} in swine (Flores et al., 1990) and avian (Asem et al., 1987) granulosa cells, bovine luteal cells (Alila et al., 1989) and rat Sertoli cells (Grasso and Reichert, 1989). Although the mechanism of FSH-stimulated Ca^{2+} transients is not known, FSH receptor-coupled Ca^{2+} channels have been proposed to be a possible route of Ca^{2+} influx since the response to the gonadotropin is dependent on the presence of extracellular Ca^{2+} (Grasso and Reichert, 1989; Flores et al., 1990). The temporal pattern of Ca^{2+} increase induced by the gonadotropin suggests an involvement of a transducing mechanism other than that associated with PIP_2 breakdown (Flores et al., 1990). LH action seems to include both intra- and extracellular Ca^{2+} mobilization and two phases of Ca^{2+} increase: one due to Ca^{2+} mobilization by IP_3 and IP_4 during PIP_2 breakdown and another believed to be mediated by receptor-operated Ca^{2+} channels and dependent on extracellular Ca^{2+}. In the bovine ovary, small and large luteal cells are known to have different Ca^{2+} requirements for their steroidogenic responses. Small luteal cells used Ca^{2+} from intra- and extracellular sources for basal and gonadotropin-stimulated steroid secretion, however large luteal cells relied only on extracellular Ca^{2+} (Alila et al., 1989). It is of interest that

8-bromo cAMP and forskolin also increased Ca^{2+} concentration in porcine granulosa cells incubated in Ca^{2+}-depleted medium (Flores et al., 1990).

D. The Cross-Talk Between Phosphatidylinositol and Adenylyl Cyclase Signal Transducing Systems

The cross-talk between signalling pathways includes second messenger-dependent systems operating through Ca^{2+}, cAMP, PKC and PKA (Nigg, 1990). In steroidogenic tissues, particular attention has been paid to the modulatory action of PKC and Ca^{2+} on the adenylyl cyclase signalling pathway. Although, PKC has been shown to have variable effects on basal and gonadotropin-stimulated cAMP formation in the ovary, the majority of reports demonstrated that TPA had no effect on basal cAMP production (Shinohara et al., 1986; Brunswig et al., 1986; Veldhuis and Demers, 1986; Wheeler and Veldhuis, 1989) and inhibited gonadotropin-induced cAMP accumulation (Mukhopadhyay and Schumacher, 1985; Asem and Tsang, 1988; Tilly and Johnson, 1988; Wheeler and Veldhuis, 1988, Hylka et al., 1989; Davis et al., 1989).

The possible regulation of the adenylyl cyclase system by PKC may be at the level of the hormone receptor (Wheeler and Veldhuis, 1988; 1989), G protein (Kelleher et al., 1984; Wheeler and Veldhuis, 1989), adenylyl cyclase (Wheeler and Veldhuis, 1989) and/or phosphodiesterase (Wheeler and Veldhuis, 1988; 1989). A positive modulation appears to include increased sensitivity of adenylyl cyclase system to agonist (e.g. forskolin) stimulation resulting in decreased ED_{50} of the latter and enhanced maximal induction of the enzyme formation (Wheeler and Veldhuis, 1989). In addition eicosanoids produced from AA during PIP_2 breakdown are known to stimulate adenylyl cyclase and amplify the signal (Bolander, 1989). The inhibitory action of PKC may include phosphorylation of the receptor, resulting in either a change of the affinity (e.g. EGF receptor) or the uncoupling of the receptor from the adenylyl cyclase (Kelleher et al., 1984). It has also been suggested that Ca^{2+}, generated during PIP_2 breakdown, binds to

calmodulin and stimulates phosphodiesterase. The latter leads to the hydrolysis of cAMP and termination of the biological response. The effect of Ca^{2+} on the adenylyl cyclase pathway appears to be biphasic: stimulatory at low (<1 μM) but inhibitory at higher concentrations of the ion (Tomlinson et al., 1985; MacNeil et al., 1985). Moreover, cAMP is known to inhibit PI kinase and PLC (O'Shea et al , 1987). These observations raise the possibility that the cyclic nucleotide also modulates the Ca^{2+} and PKC pathways in the control of steroidogenesis in ovarian cells.

THE OBJECTIVE

The main objective of the present studies was to examine the role of PKC in rat granulosa cell steroidogenesis in vitro. Specifically, the following questions were addressed:

I. Is PKC involved in the regulation of rat granulosa cell steroidogenesis in vitro? If so, which step(s) in the steroidogenic pathway is (are) PKC-dependent?

II. Is PKC involved in the stimulation of granulosa cell steroidogenesis by FSH? Does it participate in the modulation of FSH-induced steroid hormone production?

III. What are the possible reasons for the differential effects of diacylglycerols and TPA on granulosa cell steroidogenesis in vitro?

MATERIALS AND METHODS

A. Reagents

If not indicated otherwise all enzyme preparations and reagents were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.. PMSG - pregnant mares' serum gonadotropin was from Equinex, Ayerst Labs, Inc., Montreal, Quebec, Canada; MEM - Eagle's Minimal Essential Medium, containing $NaHCO_3$ (2.2g/l), non-essential amino acids (0.1 mm), penicillin-streptomycin (50,000 U/l and 50,000 µg/l respectively) and fungizone (625 µg/l) - from GIBCO Laboratories, Mississauga, Ontario, Canada; Halothane - from Superharm, St. Lambert, Quebec, Canada; FSH - NIAMDD-oFSH-14 - from National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland, U.S.A.; AGP -Aminoglutethamide phosphate, - from CIBA - Geigi Corporation, Ardsley, NY, U.S.A.; Cyanoketone-from Winthrop Laboratories, Aurora, Ontario, Canada; Dextran T-70 -from Pharmacia, AB, Uppsala, Sweden; Carbon decolorizing powder (activated) - from BDH, Darmstadt, Germany.

Before addition to the culture medium, FSH, $(Bu)_2$cAMP and forskolin were dissolved in saline and stored in small aliquots at -40° C. Phorbol esters were dissolved in acetone and also stored at -40° C. Diacylglycerols, sphingosine and H_7 were stored in chloroform under N_2 at -40° C, in aliquots adequate for single experiments. Preceding each experiment, the chloroform was evaporated (under N_2) and the PKC activators and inhibitors were dissolved by sonication (15-20 seconds) in MEM with 1% of dimethyl sulphoxide (DMSO). MEM containing H_7 was warmed up to 40° C prior to sonication, to liquefy the lipid before its emulgation. Aminoglutethamide phosphate (AGP; an inhibitor of side chain cleavage) and cyanoketone (an inhibitor 3β-hydroxysteroid dehydrogenase; Goldman et al., 1965), used in the present studies to block the production of endogenous P_5 and metabolism of P_5 to P, respectively, were dissolved in ethanol.

Also steroid hormones were dissolved in ethanol and stored at 0° C. A23187, dissolved in DMSO to form a stock solution, was stored in darkness in small aliquots at -40° C. Diacylglycerols, TPA and PKC inhibitors were similarly protected from light during all experimental procedures

Antisera for P and 20α-OH-P were kindly supplied by Drs. D.T. Armstrong and R.E. Gore-Langton, University of Western Ontario, London, Ontario, Canada. They showed negligible cross-reactivity (<0.1%) with other progestins, androgens and estrogens, with the exception of the latter which cross-reacted significantly with 20α-hydroxypregn-4-en-3-one (8.7%). The antiserum for P_5 assay, kindly provided by Dr. Gordon Niswender, Colorado State University, Fort Collins, Colorado, U.S.A., exhibited significant cross-reactivity with 17α-hydroxypregnenolone (12.7%), 5-pregnene-3β, 20α-diol (25.1%), 5-pregnene-3β, 20β-diol(8.3%) and 5-androstene-3β,17β-diol(5.6%). Aminoglutethamide phosphate and cyanoketone showed negligible cross reactivity with these antibodies (<0.1% and <0.2%, respectively).

B. Animal Preparation

The laboratory rat is a non-seasonal, spontaneously ovulating, polyestrous animal (Freeman, 1988). The typical estrous cycle is divided into four stages: proestrous, characterized by Heape (1900) as the time when animal is "coming in heat" (lasts for 12-14 hours); estrous, characterized by the willingness of the female to receive the male (25 to 27 h); metestrous, during which the changes noted in the reproductive tract at estrous subside (6 to 8 h.); and diestrous, during which ovarian secretions prepare the reproductive tract for the receipt of the ovum, fertilized shortly after mating (55 to 57 h.).

All experimental procedures described in this thesis were performed on immature, Sprague-Dawley female rats with body weight ranging between 65 - 95 g.

For induction and synchronization of follicle development, animals were injected intraperitoneally on day 28 +/-1 with a single dose of 4 IU PMSG (which has predominantly FSH activity) and were sacrificed 48 h thereafter for collection of the ovaries. With this treatment regiment, the majority of the follicles were at the antral stage of development (proestrous stage of the estrous cycle) (Fortune and Armstrong, 1977).

C. Granulosa Cell Isolation

Rats anesthetized with halothane were ovariectomized by vertical laparotomy. Ovaries, collected under aseptic conditions from animals in the proestrous stage (with enlarged, well vascularized uterus and ovaries bigger than 3.5 mm), were immediately transferred to MEM and kept on ice until further processing.

Following the removal of the bursa and the oviduct, granulosa cells were released into fresh MEM by follicle puncture and gentle squeezing of the ovary with a pair of fine forceps. The remaining tissue containing the theca cells and some granulosa cells were discarded. The granulosa cells were collected by low speed centrifugation (190 x g, 10 min.) and re-suspended in fresh MEM. Large debris sedimenting on the bottom of the centrifuge tubes was removed by a Pasteur pipette and the cell suspension was again centrifuged (190 x g, 10 min.). The pellet of granulosa cells (from 40-50 rats per one experiment) was re-suspended in 20 ml of MEM and a small aliquot was taken for cell viability determination using 0.06% trypan blue (Freshney, 1983). The number of viable cells (nonstained) and nonviable cells (stained blue) was counted on a Neubauer counting grid.

Granulosa cell preparations were treated enzymatically according to the method of Farookhi (1982) to remove the nonviable cells. Cells were incubated with trypsin (50mg/ml; 1 minute; 37° C), and subsequently with an excess of soybean trypsin inhibitor (150 mg/ml; 37° C), to stop the reaction. The DNA, released from lysed cells during the

trypsinization procedure, was digested with DNAse I (25μg/ml; 5 minutes; 37° C) to prevent clumping of the cells. The cells were then washed twice with MEM by centrifugation (190xg, 10 min) and cell viability was again determined using trypan blue. The viability of the granulosa cells (mean ± SEM; n=30 experiments) before the enzymatic treatment was 47±0.3%. Trypsinization significantly decreased total cell number, bringing down the number of nonviable cells to 3±0.04%. The viability of granulosa cells incubated with various substances, did not differ significantly at the end of incubations from that observed at the beginning.

D. Tissue Cultures

Table 1 illustrates the optimal cell density for granulosa cell steroidogenesis under in vitro conditions. A plating density of $2.55x10^5$ cells gave the best steroidogenic response to FSH, so this density was used in all subsequent experiments. Granulosa cells were resuspended in MEM and preincubated for up to 20 h (under an atmosphere of 5% CO_2 and 95% air) alone or in the presence of either OAG or TPA. At the end of the 20 h preincubation period, the medium was replaced by the fresh medium either unsupplemented or containing one or more of: FSH (0-300 ng/ml), $(Bu)_2$cAMP (1.5 mM); forskolin (1-100 μM), 1-oleoyl-2-acetylglycerol (OAG) (5-80 μg/ml), 1,2-dioctanoylglycerol (DiC_8) (5-80 μg/ml), diolein (2.5-40 μg/ml), 12-O-tetradecanoylphorbol 13-acetate (TPA) (5-80 ng/ml), phorbol-13-monoacetate (5-80 ng/ml), 4α-phorbol 12,13-didecanoate (5-80 ng/ml), 1-(5-Isoquinolinylsulfonyl)-3-methylpiperazine free base (H_7, 10^{-5}-10^{-3} μM), DL-erythrosphingosine (ESP, 1-10 μM), calcium ionophore (A23187, 0.125-1ng/ml), P_5 (0,1 μM) and P (0,1 μM). All experimental groups contained the same concentrations of the vehicles (0.08% of acetone and 1% of DMSO). Granulosa cells were incubated for 0,1,3,6,12 and 24 h, depending

Table 1 Influence of granulosa cell plating density on basal, FSH (150 ng/ml)- and TPA (40 ng/ml)-stimulated secretion of P during a 24 h culture period.

Plating density (10^5 cells per well)	Secretion of P (% of control)		
	Control	FSH	TPA
1.27	100 ±10	300 ±23	56 ±4
2.55	100 ±9	406 ±31	55 ±8
3.82	100 ±14	266 ±11	47.3 ±8
5.00	100 ±15	195±38%	52.4%

Results are normalized to ng/10^5 cells and are expressed as a percentage of their respective controls (Control - defined as 100%). Values represent means ± SEM of 8 incubations from 2 experiments.

on the experimental design. At the end of each culture period the medium was collected and stored at -20° C pending steroid analysis.

E. Extraction and Radioimmunoassay Measurement of Steroid Hormones Secretion

At the end of the incubation period steroid hormones secreted into the medium (1 ml) were extracted twice with 3 ml of 95% anhydrous diethyl ether. The media were vortexed for 2 minutes and dipped quickly in a nitrogen-cooled ethanol bath. The organic phase containing the steroids was decanted from the frozen aqueous phase to another set of glass tubes. The media were extracted again (for 1 min) and the two ether extracts from each sample were combined and evaporated to dryness under air. The samples were redissolved in 1 ml of redistilled ethanol and stored in tightly closed tubes.

To determine the percent recovery of each steroid during extraction, two additional tubes each containning 1 ml MEM and a known amount of tritium-labelled hormone were extracted along with unknown samples. A correction factor, based on the percentage of recovery of the labelled steroid, was calculated and applied to each sample analyzed. The recovery was within the range of 97 -99 %.

To measure the concentration of steroid hormones in granulosa cells preincubated for 20 h, the MEM in each well was replaced by 1 ml of 80% ethanol; the cells were scraped with rubber policeman and their suspensions centrifuged. Steroid levels in the supernatant were determined by radioimmunoassay.

Pregnenolone (P_5), progesterone (P), and 20α-hydroxypregn-4-en-3-one (20α-OH-P) in the extracts were measured at 4° C by specific radioimmunoassays (Orczyk et al., 1979; Inaba and all, 1980). These assays were carried out in a phosphate buffered saline-gelatin solution [PBSG: sodium phosphate, dibasic (10.79 g/L); sodium phosphate, monobasic (2.39 g/L); EDTA (0.37 g/L); sodium azide (0.19 g/L); sodium

chloride (9.0 g/L) and gelatin (1.0 g/L)] at pH 6.9. The total assay volume was 500 µl. the ethanolic solutions of the standards and samples evaporated to dryness under air and resuspended in 100 µl of PBSG, 100 µl of tritiated P_5, P or 20α-OH-P (~15,000 counts per minute; in PBSG) and 100 µl of an appropriate antibody (at a concentration to bind 30% of the radio-labelled steroid).

All samples were measured in duplicate. The standard curve for each assay included triplicate tubes for the determination of: (a) the total amount of radioactivity added to each tube (total count); (b) the amount of tritiated steroid bound to the antibody in the absence of unlabeled steroid; (c) non-specific binding and (d) the amount of tritiated steroid bound in the presence of various known concentrations of progestin (5,10,20,40,80,160,320 and 640 pg). To determine the inter- and intra-assay variations, each radioimmunoassay also included triplicate determinations of steroid pools with known high (300 pg/100 µl) and low (25 pg/100 µl) concentrations.

The assay tubes were vortexed gently and left overnight to equilibrate at 4° C, a temperature optimal for competitive binding of labelled and unlabeled ligand (steroid) to the antibody (Yallow, 1985). The following morning, steroid hormones not bound specifically to the antibody were separated by absorption to dextran-coated charcoal. The assay tubes were placed in an ice bath and 0.7 ml of charcoal mixture [containing Dextran T-70 (0.357 g/L) and carbon decolorizer (3.57 g/L) in phosphate buffered saline] were added to each tube (except those for "total counts" to which 0.7 ml of phosphate buffer saline was added instead). After 15 minutes, the assay tubes were centrifuged (550 x g for 15 minutes) to separate bound and free steroids. Supernatant, containing the steroid-antibody complex, was decanted into scintillation vials and scintillation fluid (3.5 ml) (glacial acetic acid, 5 ml/L toluene, 2,5-dephenyl-oxazole, 7 g/L of toluene) was added. After 4 hours of equilibration at room temperature (to facilitate the extraction of steroid from the aqueous phase to the toluene layer), the radioactivity in the vials was determined over a 3 minute period, in a 1218 Rackbeta Liquid Scintillation counter (LKB Wallac).

The sensitivities of the radioimmunoassays for P_5, P and 20α-OH-P measurements were 20 pg, 5 pg and 5-10pg respectively. The inter- and intra-assay variations for P_5 (in the presence of cyanoketone), P and 20α-OH-P were: 18% and 10%, 17% and 8%, and 20% and 9% respectively. An example of P standard curve plotted using spline function interpolation is shown in Fig. 9.

E. Statistical Analysis

Results were assessed by one or two-way analysis of variance. When required, data were transformed logarithmically prior to statistical analyses to remove heterogeneity of variance as determined by Bartlett's test. Significant differences between treatment groups were determined by Duncan's new multiple range test. Statistical significance was inferred at $p< 0.05$. Data are the mean±SEM.

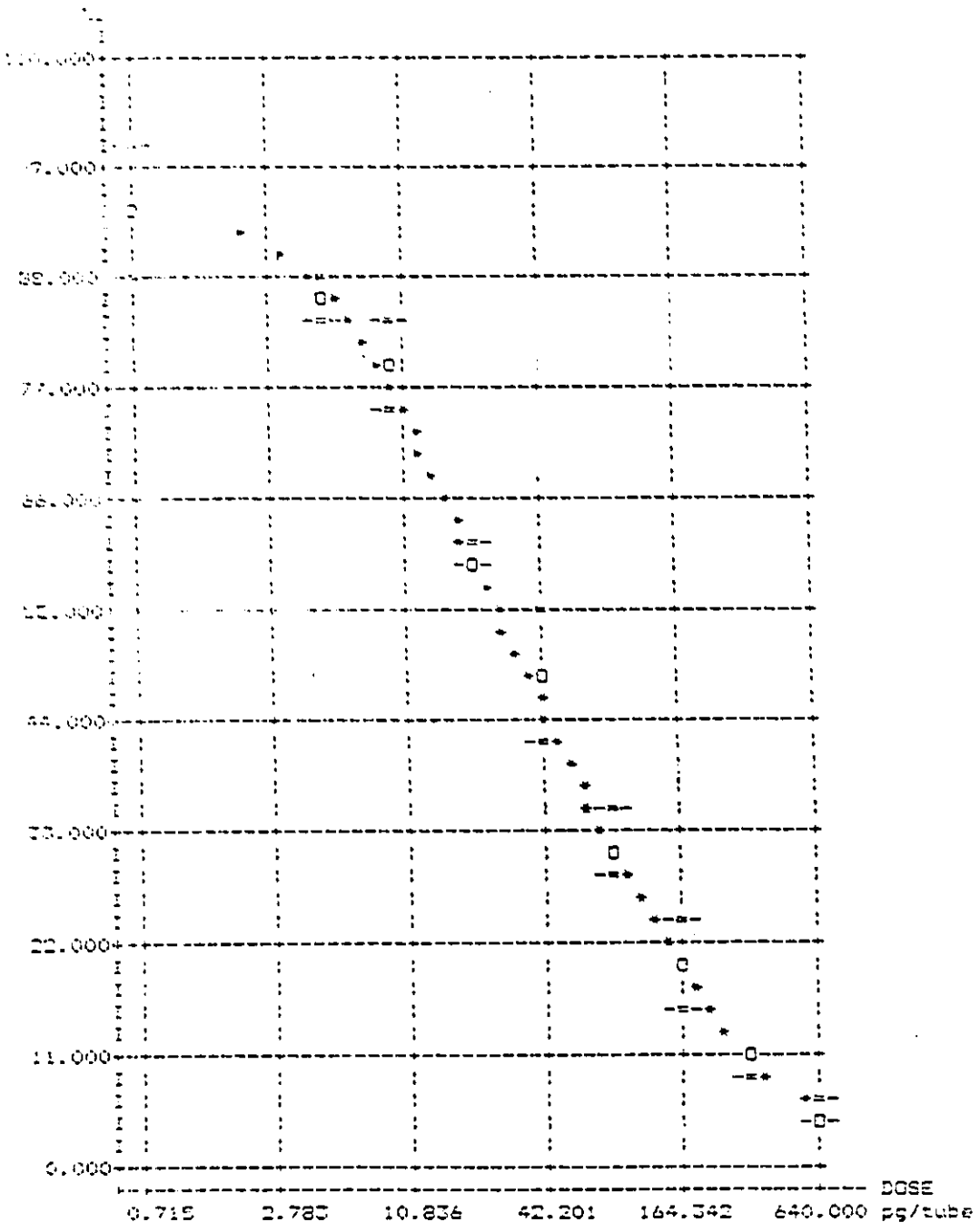

Figure 9 Standard curve for progesterone - spline fit.

RESULTS

I. IS PKC INVOLVED IN THE REGULATION OF RAT GRANULOSA CELL STEROIDOGENESIS IN VITRO? IF SO, WHICH STEP(S) IN THE STEROIDOGENIC PATHWAY IS (ARE) PKC DEPENDENT?

The involvement of PKC in the regulation of granulosa cell steroidogenesis was examined by measuring the levels of steroid hormones secreted by these cells incubated in the presence of PKC activators (diacylglycerols, tumor promoting phorbol esters) and inhibitors [isoquinolinylsulfonyl derivative (H_7) and D-L-erythrosphingosine (ESP)].

A. Influence of Synthetic Diacylglycerols and Phorbol Esters on Basal Secretion of Steroids.

1) Dose-Dependent Effects of OAG, DiC_8, Diolein, TPA, 4α-Phorbol 12,13-Didecanoate and Phorbol-13-Monoacetate on Basal, P_5, P and 20α-OH-P Secretion.

P_5 secretion by granulosa cells incubated for 12 h in the absence of hormonal or nonhormonal agonists was low (<0.05 ng/10^5 cells; Table 2). Addition of OAG (5-80 μg/ml) or TPA (5-80 ng/ml) had no significant ($p>0.05$) effect on the secretion of the steroid. OAG (5-80 μg/ml) and DiC_8 (5-80 μg/ml), however, significantly ($p<0.05$) and dose-dependently increased both P and 20α-OH-P secretion (Fig. 10, Table 3). The minimal concentration of OAG significantly ($p<0.05$) increasing P and 20α-OH-P secretion was 10 μg/ml, and minimal concentrations of DiC_8 were 40 μg/ml and 5 μg/ml, respectively. Maximal stimulation of total progestin (P+20α-OH-P) secretion was noted at 10μg/ml of OAG (~200%) and 40-80 μg/ml of DiC_8 (~300%) (Fig. 10, Table 3). The progestin secretion ratio (calculated to determine the rate of conversion of P to 20α-OH-P) was slightly but significantly ($p<0.05$) inhibited by higher concentrations (20-80 μg/ml)

of OAG during the 12 h incubation period (Table 4), but was increased by DiC_8 (10-160 μg/ml) during 24 h cultures (Tables 4 and 5). As expected, diolein (2.5-40μg/ml), the nonpermeable diacylglycerol, did not have any effect on P and 20α-OH-P secretion (Table 3).

TPA (5-80 ng/ml) significantly enhanced the secretion of 20α-OH-P and total progestins (P+20α-OH-P) but not of P (Fig. 11). The minimal and maximal stimulatory concentrations of TPA on 20α-OH-P and total steroid secretion were 10 ng/ml and 40 ng/ml, respectively. The 20α-OH-P/P secretion ratio was significantly ($p<0.05$) increased by all concentrations of TPA (Table 4). Two phorbol esters with no tumor promoting activity, phorbol-13-monoacetate and 4α-phorbol 12,13-didecanoate, failed to significantly influence P_5, P and 20α-OH-P secretion (Table 6).

Table 2 Dose-dependent effects of OAG and TPA on basal P_5 secretion during a 12 h incubation period.

Treatment Groups	P_5 secretion (ng/10^5 cells)
Control	0.023 ± 0.006
OAG (5 µg/ml)	0.023 ± 0.007
OAG (20 µg/ml)	0.038 ± 0.009
OAG (80 µg/ml)	0.038 ± 0.012
TPA (5 ng/ml)	0.021 ± 0.002
TPA (40 n/ml)	0.027 ± 0.005
TPA (80 ng/ml)	0.018 ± 0.006

Values represent means ± SEM of 9 incubations from 3 experiments.

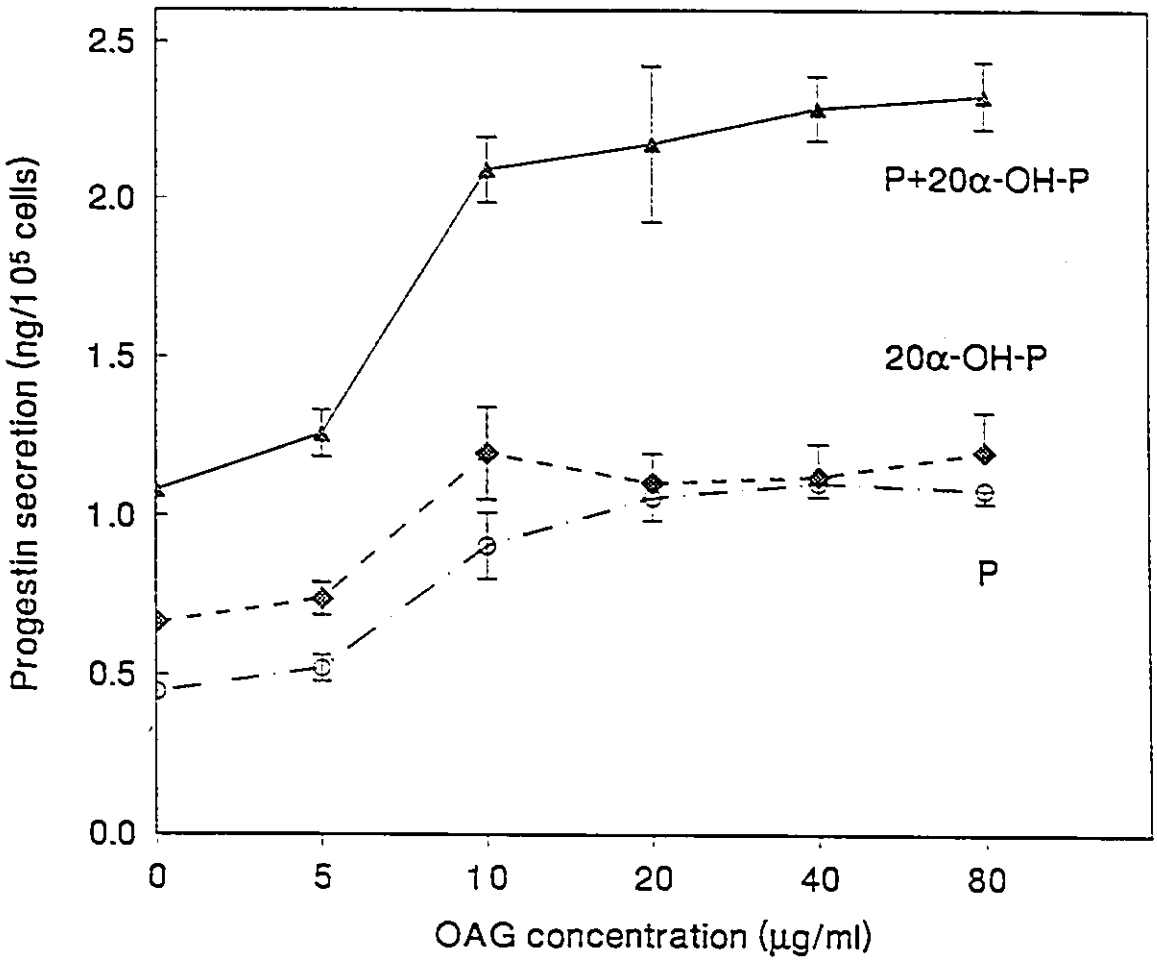

Figure 10 Dose-dependent effect of OAG on secretion of P, 20α-OH-P and P+20α–OH-P by rat granulosa cells during a 12 h incubation period. Values represent means ±SEM of 9 incubations from 3 experiments.

Table 3 Concentration-dependent effects of DiC_8 and diolein (Diol) on P and 20α - OH-P secretion during a 24 h culture period.

Treatment Groups	Steroid Secretion (ng/10^5 cells) P	20α - OH-P
Control	0.25 ± 0.01	0.43 ± 0.01
DiC_8 (5µg/ml)	0.27 ± 0.01	0.72 ± 0.07 a
DiC_8 (10µg/ml)	0.26 ± 0.01	0.77 ± 0.07 a
DiC_8 (20µg/ml)	0.28 ± 0.01	0.99 ± 0.11 a
DiC_8 (40µg/ml)	0.30 ± 0.01 a	1.10 ± 0.11 a
DiC_8 (80µg/ml)	0.35 ± 0.01 a	1.20 ± 0.11 a
DiC_8 (160µg/ml)	0.35 ± 0.01 a	0.92 ± 0.01 a
Control	0.27 ± 0.02	0.95 ± 0.10
Diol (2.5µg/ml)	0.26 ± 0.03	1.06 ± 0.09
Diol (5µg/ml)	0.29 ± 0.03	1.03 ± 0.13
Diol (10µg/ml)	0.32 ± 0.03	1.09 ± 0.18
Diol (20µg/ml)	0.34 ± 0.04	1.14 ± 0.12
Diol (40µg/ml)	0.20 ± 0.03	0.83 ± 0.07

Values represent means ± SEM of 9 incubations from 3 experiments.

a $p < 0.05$ (compared to control)

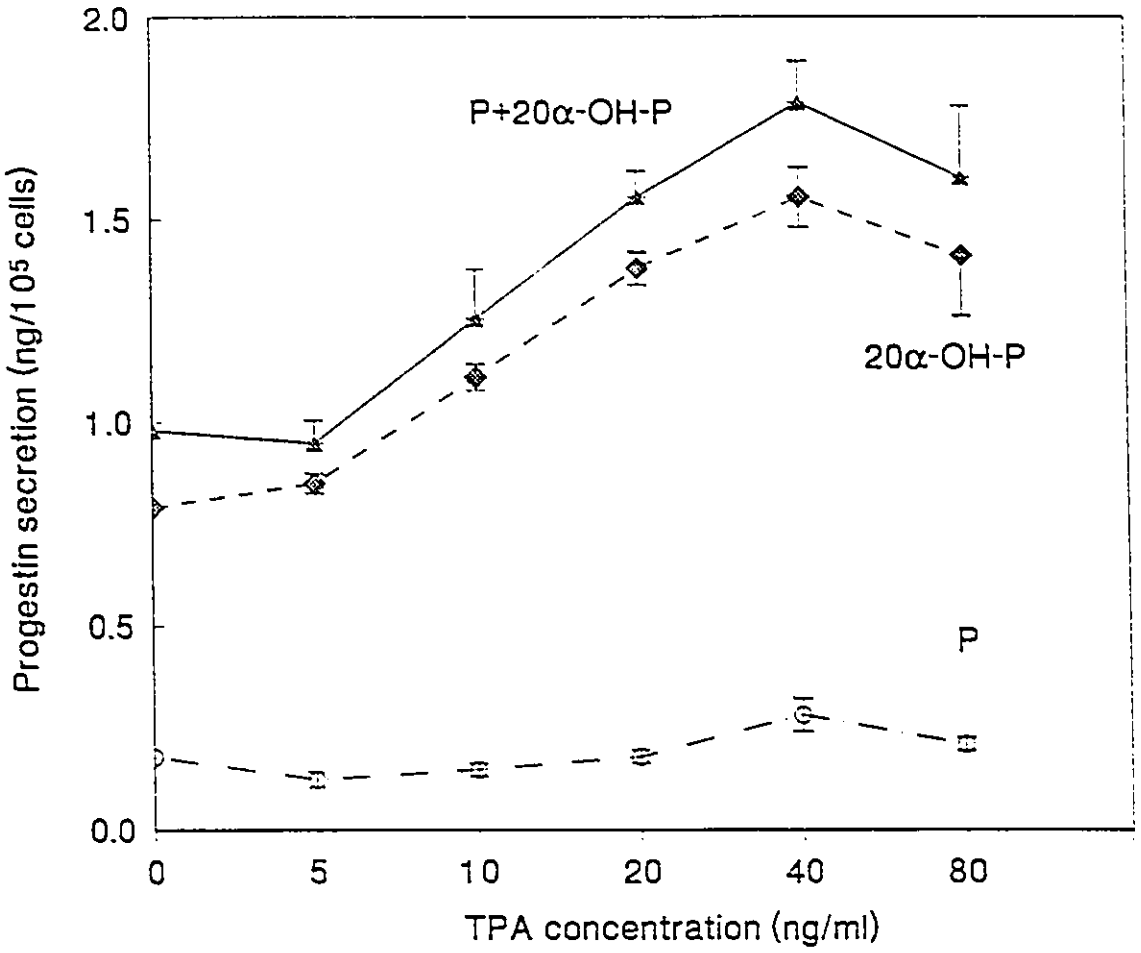

Figure 11 Dose-dependent effect of TPA on secretion of P, 20α-OH-P and P+20α–OH-P by rat granulosa cells during a 12 h incubation period. Values represent means ±SEM of 13 incubations from 3 experiments.

Table 4 Concentration-dependent effects of OAG and TPA on 20α-OH-P/P secretion ratio during a 12 h incubation period.

Treatment Groups	20α - OH-P/P Secretion Ratio
Control	100 ± 5%
OAG (5µg/ml)	103 ± 6%
OAG (10µg/ml)	100 ± 11%
OAG (20µg/ml)	68 ± 4% [a]
OAG (40µg/ml)	71 ± 4% [a]
OAG (80µg/ml)	74 ± 3% [a]
Control	100 ± 3%
TPA (5ng/ml)	160 ± 11% [a]
TPA (10ng/ml)	185 ± 15 % [a]
TPA (20ng/ml)	178 ± 19% [a]
TPA (40ng/ml)	139 ± 12% [a]
TPA (80ng/ml)	168 ± 21% [a]

Results are expressed as means ± SEM of 9 incubations from 3 experiments, each calculated as a % of the respective control value defined as 100%. The 20α-OH-P/P secretion ratios for the control group in the OAG and TPA experiments are 1.65 ± 0.20 and 2.88 ± 0.28, respectively.

[a] - $p < 0.05$ (compared to control)

Table 5 Concentration-dependent effects of DiC_8 on 20α-OH-P/P secretion ratio during a 24 h incubation period.

Treatment Groups	20α - OH-P/P Secretion Ratio
Control	100 ± 5%
DiC_8 (5µg/ml)	100 ± 2%
DiC_8 (10µg/ml)	157 ± 10% [a]
DiC_8 (20µg/ml)	174 ± 10% [a]
DiC_8 (40µg/ml)	205 ± 11% [a]
DiC_8 (80µg/ml)	213 ± 9% [a]
DiC_8 (160µg/ml)	153 ± 11% [a]
Control	99 ± 10%
Diol (2.5µg/ml)	103 ± 10%
Diol (5µg/ml)	101 ± 13 %
Diol (10µg/ml)	97 ± 16%
Diol (20µg/ml)	95 ± 10%
Diol (40µg/ml)	118 ± 10%

Results are expressed as means ± SEM of 9 incubations from 3 experiments, each calculated as a % of the respective control value defined as 100%. The 20α-OH-P/P secretion ratios for the control group in the DiC_8 and Diol experiments are 1.73 ± 0.12 and 3.50 ± 0.28, respectively.

[a] - $p < 0.05$ (compared to control)

Table 6 Lack of effects of 4α-phorbol 12,13-didecanoate and phorbol-13-monoacetate on P_5, P and 20α-OH-P secretion during a 12 h incubation period.

	Steroid Secretion (ng/10^5 cells)		
Treatment Groups	P_5	P	20α - OH-P
Control	1.11 ± 0.22	0.33 ± 0.02	0.79 ± 0.06
Phorbol-13-monoacetate			
5 ng/ml	1.02 ± 0.21	0.40 ± 0.03	0.82 ± 0.06
40 ng/ml	1.15 ± 0.15	0.32 ± 0.02	0.91 ± 0.11
80 ng/ml	1.21 ± 0.20	0.39 ± 0.03	0.85 ± 0.07
4α-phorbol 12,13-didecanoate			
5 ng/ml	0.98 ± 0.17	0.32 ± 0.02	0.76 ± 0.05
40 ng/ml	1.05 ± 0.12	0.38 ± 0.04	0.69 ± 0.07
80 ng/ml	1.12 ± 0.08	0.29 ± 0.01	0.82 ± 0.05

Values represent means ± SEM of 9 incubations from 3 replicate experiments.

2) Time Course of the Effects of OAG and TPA on Basal Secretion of P_5, P and 20α-OH-P.

To determine the time course of action of PKC activators, granulosa cells were cultured for 0,1,3,6,12 and 24 hours with fresh MEM containing a maximal stimulatory concentration of either OAG (20 μg/ml) or TPA (40 ng/ml). As shown in Figs 12 - 15, significant ($p<0.05$) enhancement of P_5 (in the presence of 25 μM of cyanoketone) and total progestin (P+20α-OH-P) secretion was noted as early as 6 h of incubation with OAG (20 μg/ml). Secretion of the steroids continued to increase thereafter, reaching the maximum for P_5 (200%), P, 20α-OH-P (180% - 190%) and P+20α-OH-P (190%) between 12 - 24 h of incubation. The effects of TPA (40 ng/ml) on P_5 and P secretion were biphasic. TPA significantly ($p<0.05$) increased the secretion of these steroids by 30% (P_5) and 200% (P) at 3 h, but inhibited their secretion by 50% at 24 h. 20α-OH-P secretion was significantly ($p<0.05$) stimulated at 3 h and thereafter, with maximal effect (200%) at 6 h - 12 h of culture (Fig. 14). 20α-OH-P/P secretion ratio in the presence of OAG remained unchanged within 24 h of incubation, although it was higher than control during the first three hours. TPA, however, significantly increased the steroid secretion ratio at 12 and 24 h of culture (Table 7).

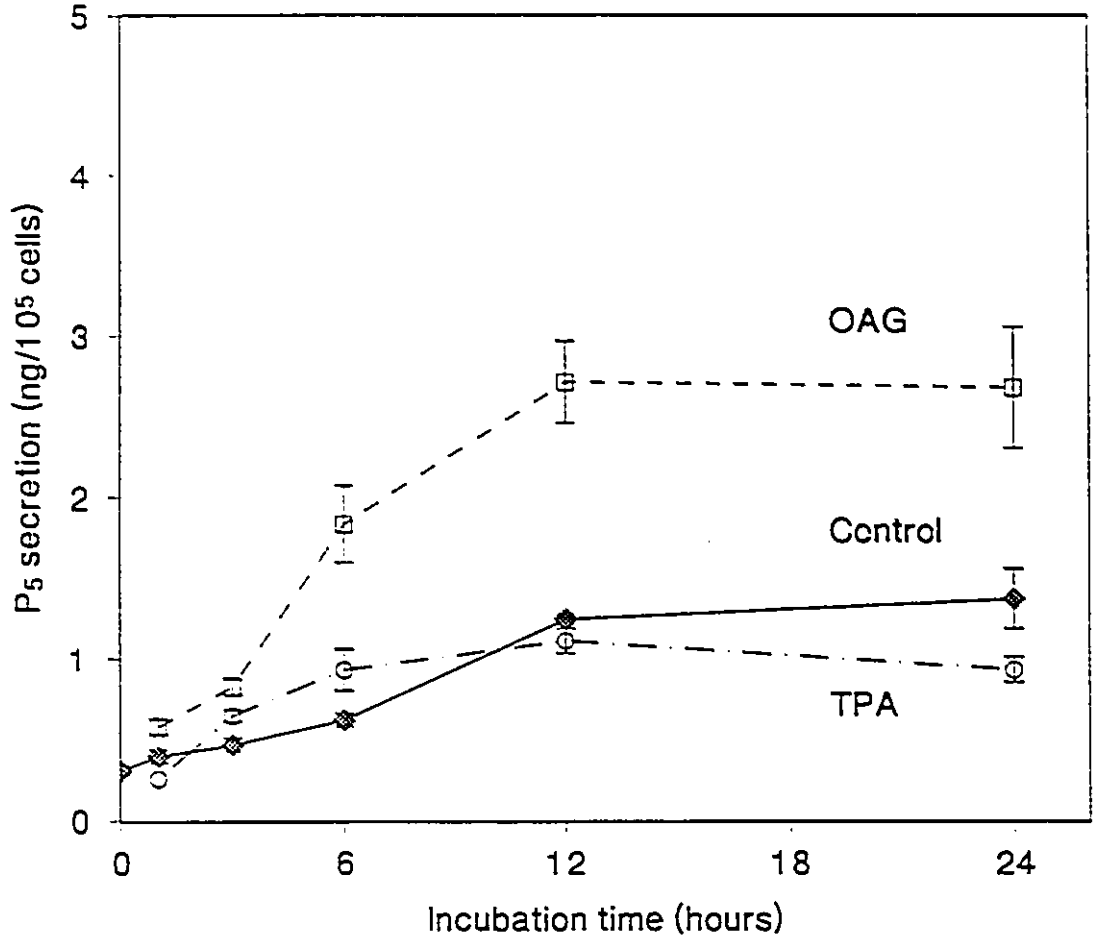

Figure 12 Time course of the effects of OAG (20 µg/ml) and TPA (40 ng/ml) on secretion of P_5 in the presence of 25 µM of cyanoketone. Values represent means ±SEM of 6 incubations from 2 experiments.

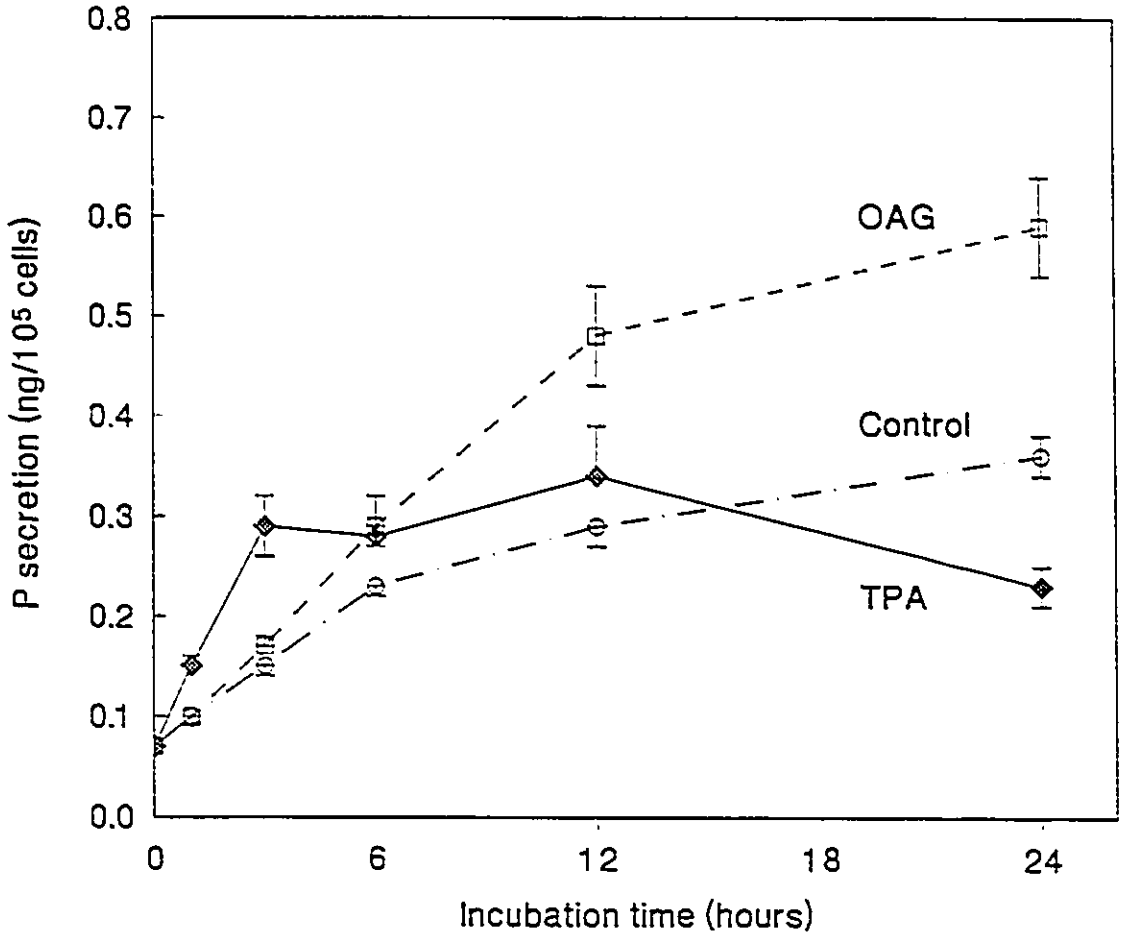

Figure 13 Time course of the effects of OAG (20 µg/ml) and TPA (40 ng/ml) on secretion of P. Values represent means ±SEM of 18 incubations from 6 experiments.

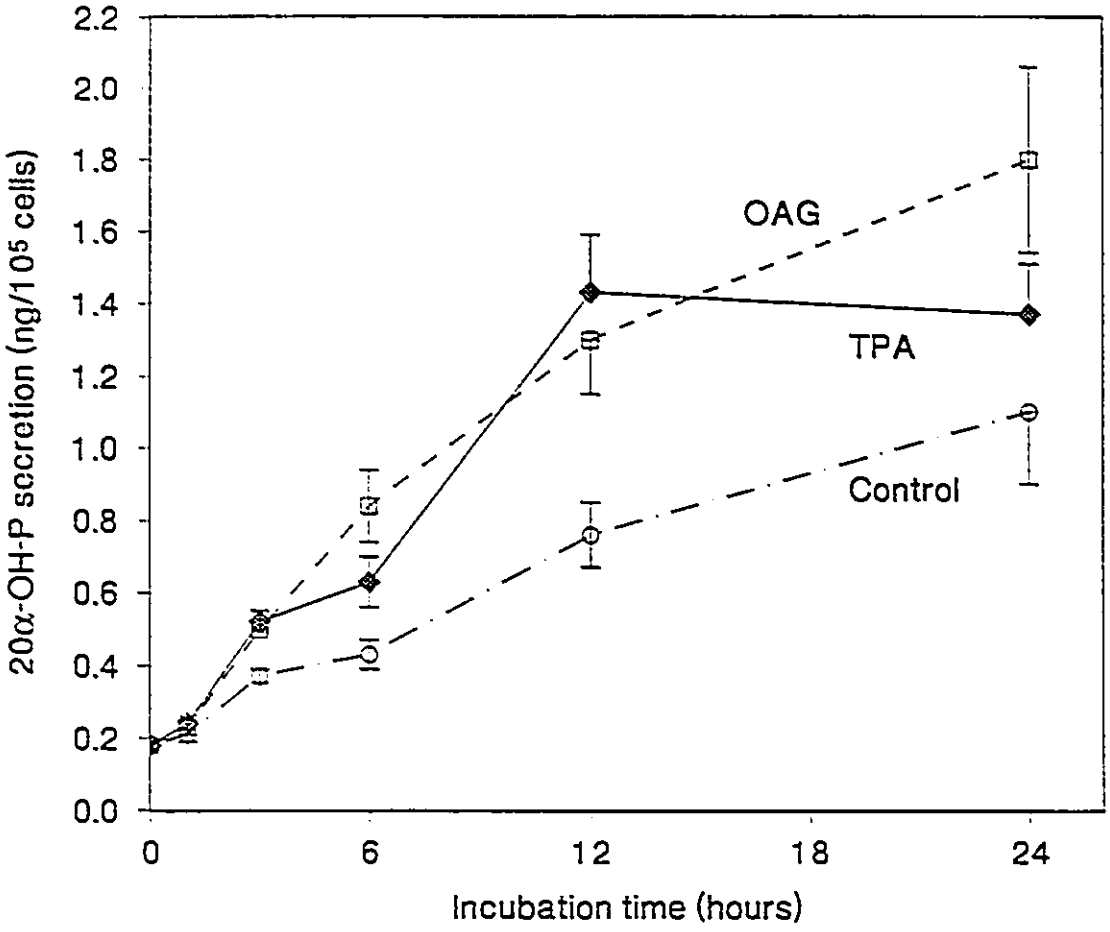

Figure 14 Time course of the effects of OAG (20 µg/ml) and TPA (40 ng/ml) on secretion of 20α–OH-P. Values represent means ±SEM of 18 incubations from 6 experiments.

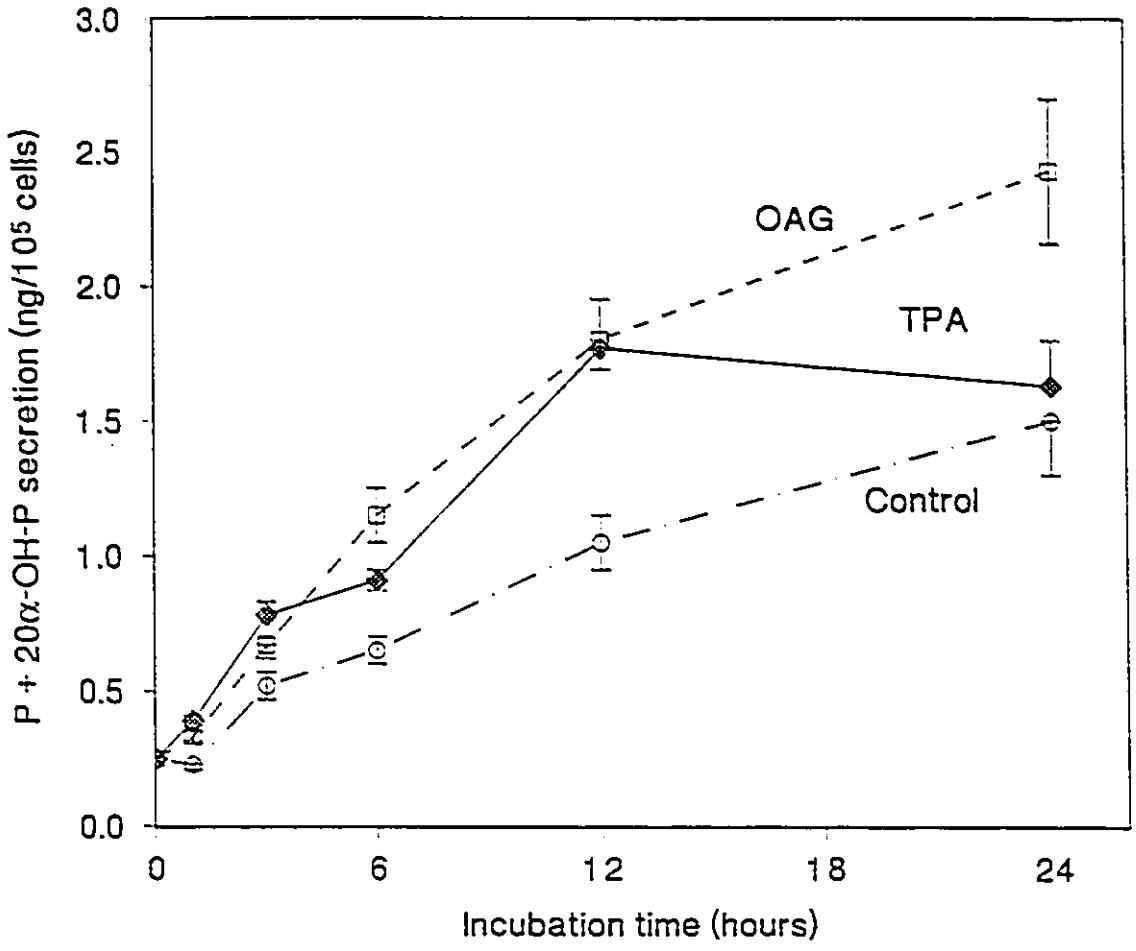

Figure 15 Time course of the effects of OAG (20 µg/ml) and TPA (40 ng/ml) on secretion of total progestins (P+20α–OH-P). Values represent means ±SEM of 18 incubations from 6 experiments.

Table 7 Time-course study of the effects of OAG (20 μg/ml) and TPA (40 ng/ml) on 20α-OH-P/P secretion ratio.

	20α - OH-P/P Secretion Ratio					
Treatment groups	0 h	1 h	3 h	6 h	12 h	24 h
Control	2.0 ± 0.2	2.2 ± 0.2	2.6 ± 0.2	2.3 ± 0.3	2.6 ± 0.2	3.2 ± 0.2
OAG (20 μg/ml)	2.0 ± 0.2	3.4 [a] ± 0.3	3.6 [a] ± 0.3	2.9 ± 0.4	2.9 ± 0.3	3.4 ± 0.3
TPA (40 ng/ml)	2.0 ± 0.2	2.4 ± 0.3	2.2 ± 0.2	2.2 ± 0.3	4.2 [a] ± 0.5	5.9 [a] ± 0.5

Values represent means ± SEM of 18 incubations from 6 experiments.

[a] $p < 0.05$ (compared to control)

B. Influence of PKC Inhibitors on OAG- and TPA-Stimulated Steroidogenesis.

To further assess whether the effects of OAG and TPA might be mediated through the activation of PKC, granulosa cells were incubated first for 1 h with H_7 or ESP and subsequently for an additional 6 h in the presence of the inhibitors and OAG (20 μg/ml) or TPA (40 ng/ml). Both PKC inhibitors significantly ($p<0.05$) suppressed the stimulatory effects of TPA and OAG in a concentration-dependent manner. Complete inhibition of the OAG- and TPA- induced responses was noted at 10^{-4} M of H_7 and 10^{-5} M of ESP (Figs. 16 and 17). At these concentrations neither H_7 nor ESP significantly suppressed basal progestin secretion by the granulosa cells. Higher concentrations of the inhibitors (10^{-3} M of H_7 and 10^{-5} M of ESP) inhibited steroid secretion and reduced cell viability (data not shown).

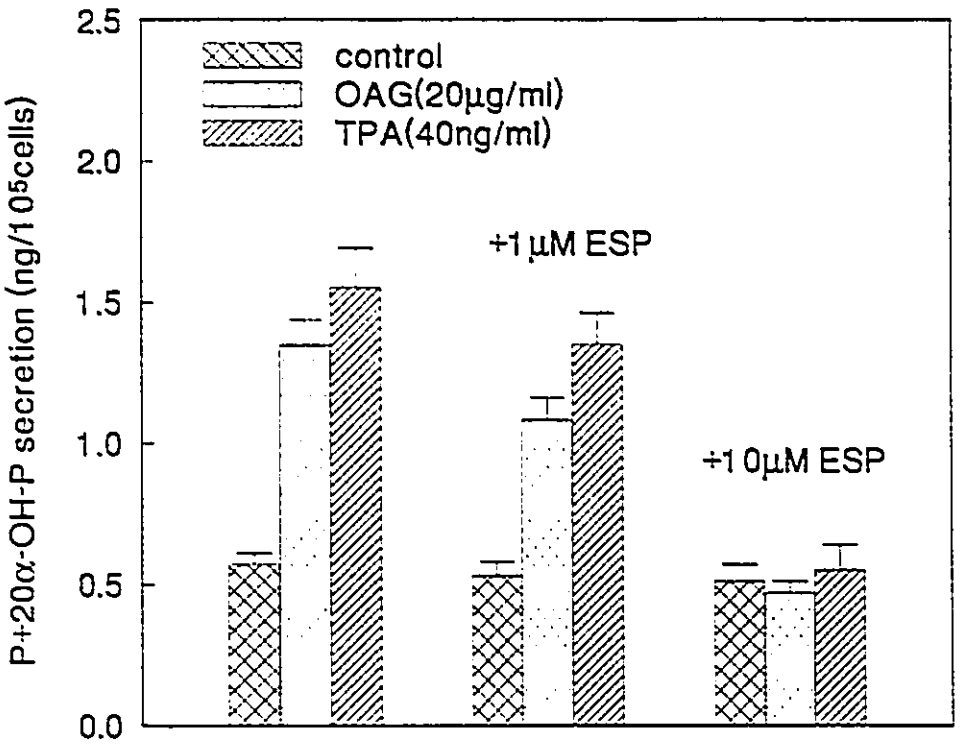

Figure 16 Concentration-dependent action of ESP on OAG (20 µg/ml)- and TPA (40 ng/ml) - stimulated secretion of total progestins (P+20α-OH-P) during a 6 h incubation period. Values represent means ±SEM of 6 incubations from 2 experiments. Addition of ESP was 1 h prior to that of OAG and TPA.

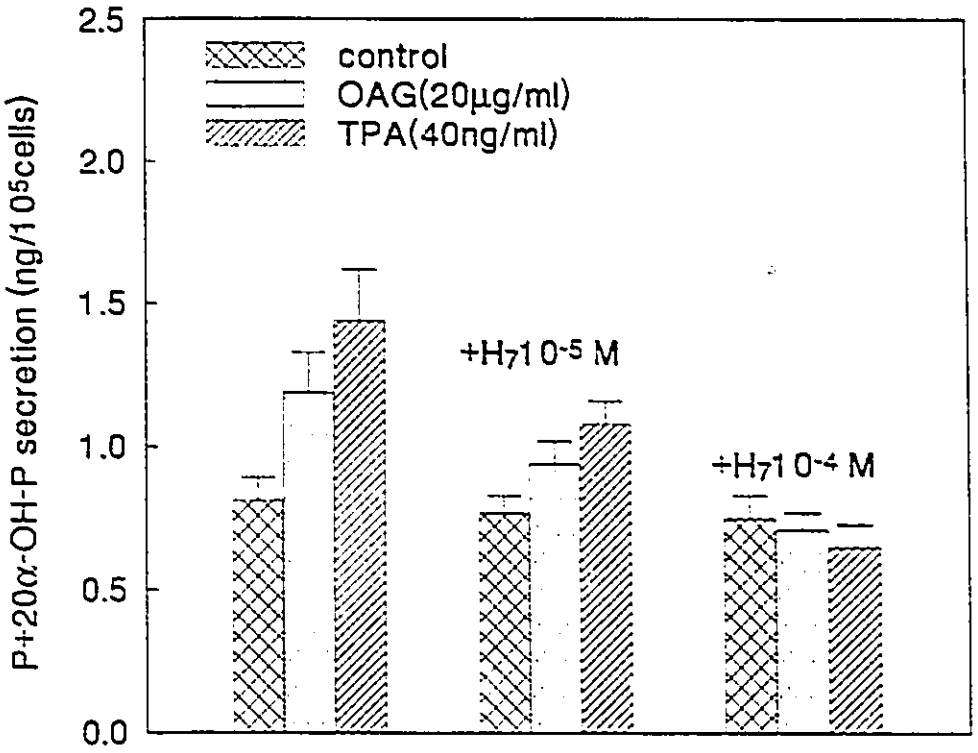

Figure 17 Concentration-dependent action of H_7 on OAG (20 μg/ ml)- and TPA (40 ng/ml)- stimulated secretion of total progestins (P+20α–OH-P) during 6 h cultures. Values represent means ±SEM of 6 incubations from 2 experiments. Addition of H_7 was 1 h prior to that of OAG and TPA.

C. Influence of OAG and TPA on Separate Steps on the Steroidogenic Pathway.

To determine the step(s) on the steroidogenic pathway which is(are) PKC-dependent, granulosa cells were incubated with OAG or TPA in the presence of specific inhibitors of steroidogenic enzymes. Separation of the steroidogenic steps by these inhibitors enabled an indirect assessment of the influence of various PKC-specific agents on the enzymes involved in the conversion of cholesterol to P_5, P_5 to P, and P to 20α-OH-P P_5 accumulation was determined in the presence of cyanoketone (an inhibitor of 3β-hydroxysteroid dehydrogenase) to prevent the metabolism of P_5 to P (Fig. 18). The ability of granulosa cells to convert P_5 to total progestins (P+20α-OH-P) was assessed in the presence of exogenous P_5 and AGP (an inhibitor of $P_{-450scc}$) to avoid the contribution from endogenous P_5 (Fig. 18). Finally, conversion of P to 20α-OH-P by 20α-HSD was determined in the presence of cyanoketone (to block endogenous P production) and exogenous P.

Several concentrations of P_5 and P were initially tested in the granulosa cell systems and 0.01 μM of both steroids was adopted as a standard condition. This concentration was within the physiologic range in the follicle and did not interfere with the radioimmunoassay used in the present studies.

1) Dose-Dependent Effects of OAG and TPA on P_5 Secretion in the Presence of Cyanoketone.

Granulosa cells were incubated for 6 h with OAG (5-80 μg/ml) or TPA (5-80ng/ml) in the presence of 25 μM of cyanoketone. Both OAG and TPA significantly ($p<0.05$) stimulated secretion of P_5 from endogenous cholesterol (Fig. 19). The effect of OAG was biphasic, with maximal stimulation (~300%) observed at 10 -20 μg/ml. At a maximal stimulatory concentration (40 ng/ml), TPA increased P_5 secretion by 40% ($p<0.05$).

2) Dose-Dependent Effects of OAG and TPA on Conversion of Exogenous P_5 to P and 20α-OH-P in the Presence of AGP

Granulosa cells were incubated with OAG (5-80 μg/ml) or TPA (5-80ng/ml) for 24 h in the presence of 0.75 mM AGP. OAG exerted a biphasic effect on total progestin (P+20α-OH-P) secretion with maximal stimulation of P (460%) and 20α-OH-P (300%) secretion noted at 10 μg/ml (Fig. 20). It had no significant influence on 20α-OH-P/P secretion ratio (Fig. 20) except at the highest concentration (80 μg/ml), which was inhibitory.

The metabolism of exogenous P_5 to P was significantly ($p<0.05$) decreased by TPA (~50%) whereas that to 20α-OH-P was significantly ($p<0.05$) enhanced by 10 ng/ml of the phorbol ester, which augmented the conversion of P_5 to 20α-OH-P by 50% (Fig. 21). 20α-OH-P/P secretion ratio was markedly ($p<0.05$) increased by TPA (Fig. 21).

3) Dose-Dependent Effects of OAG and TPA on Conversion of Exogenous P to 20α-OH-P in the Presence of Cyanoketone

Granulosa cells were cultured for 24 h with OAG (2.5-80μg/ml) or TPA (2.5-80 ng/ml) in the presence of 25 μM of cyanoketone. Both OAG and TPA significantly ($p<0.05$) increased the conversion of P to 20α-OH-P (Fig. 22). The effect of OAG was biphasic, with maximal stimulation (170%) noted at 5-10 μg/ml. Higher concentrations of OAG (eg. 80μg/ml) were less effective. The stimulatory action of TPA reached a plateau at a concentration of 5 ng/ml.

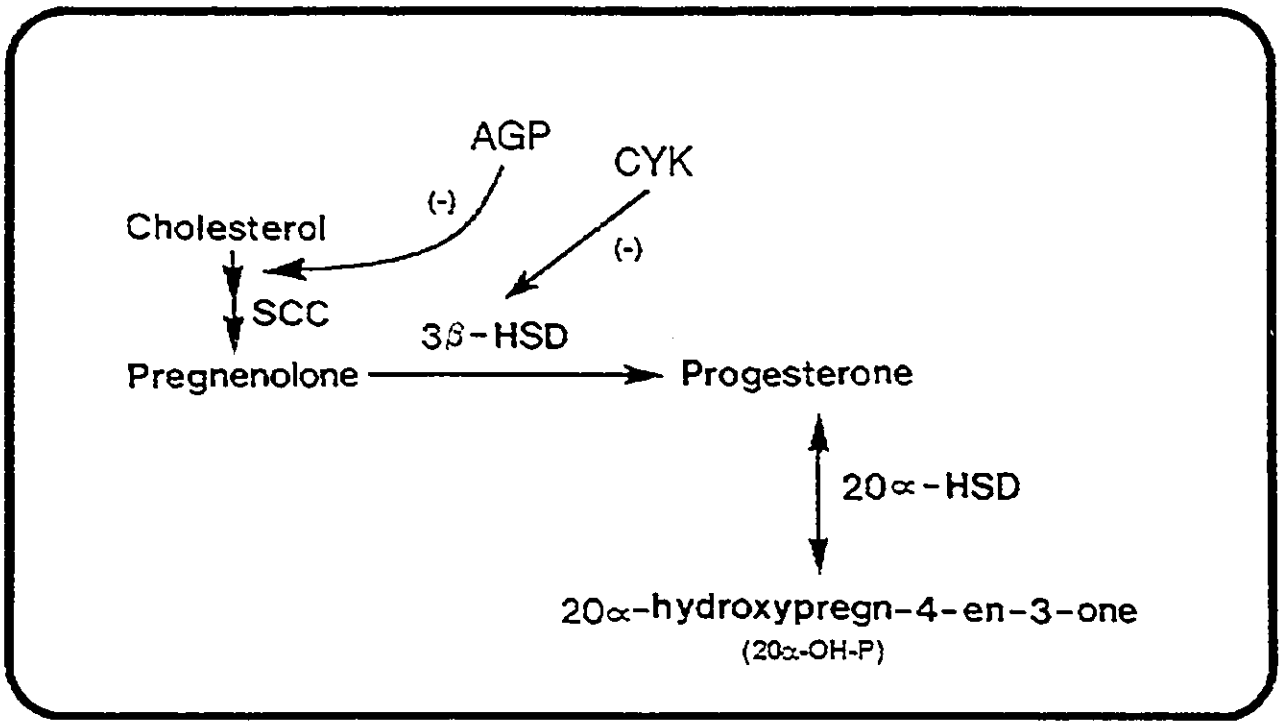

Figure 18 Sites of inhibition of progestin synthesis in granulosa cells by aminoglutethamide phosphate (AGP) and cyanoketone (CYK).

SCC - cholesterol side chain cleavage enzyme
3β–HSD - 3β-hydroxysteroid dehydrogenase
20α-HSD 20α-hydroxysteroid dehyrogenase

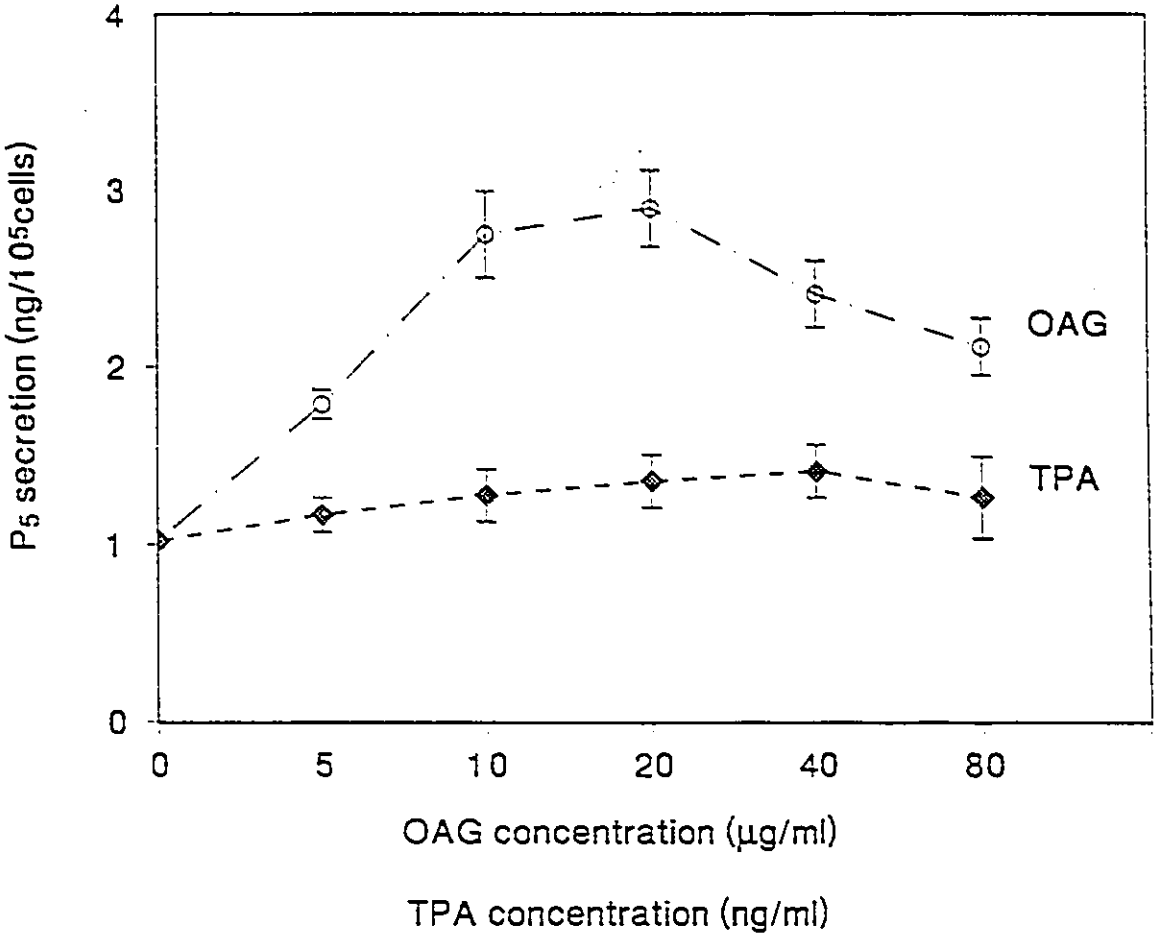

Figure 19 Concentration-dependent effects of OAG and TPA on P_5 secretion (in the presence of 25 µM cyanoketone) during a 6 h incubation period. Values represent means ±SEM of 6 incubations from 2 experiments.

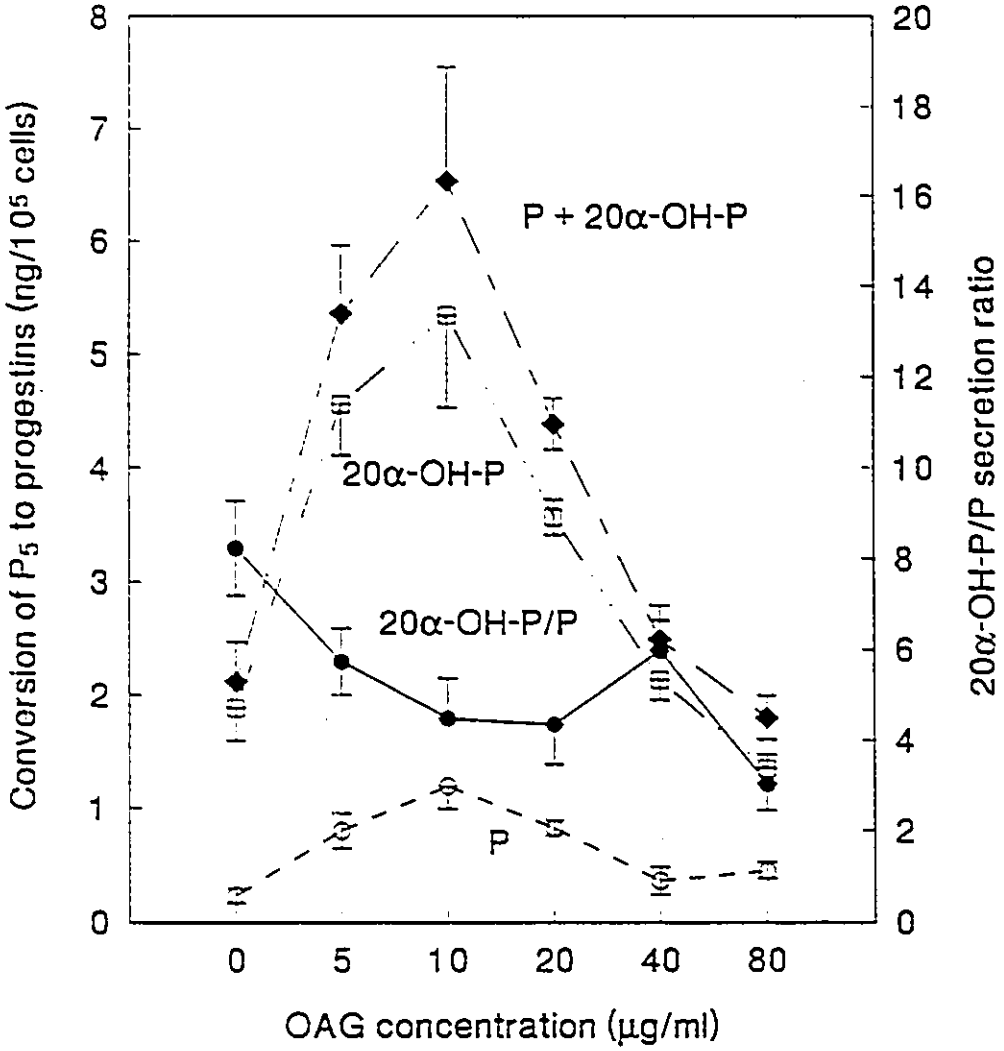

Figure 20 Concentration-dependent influence of OAG on the conversion of exogenous P_5 (0.1 µM) to P, 20α-OH-P and P+20α-OH-P (in the presence of 0.75 mM AGP) during a 24 h incubation period. Values represent means ±SEM of 10 incubations from 3 experiments.

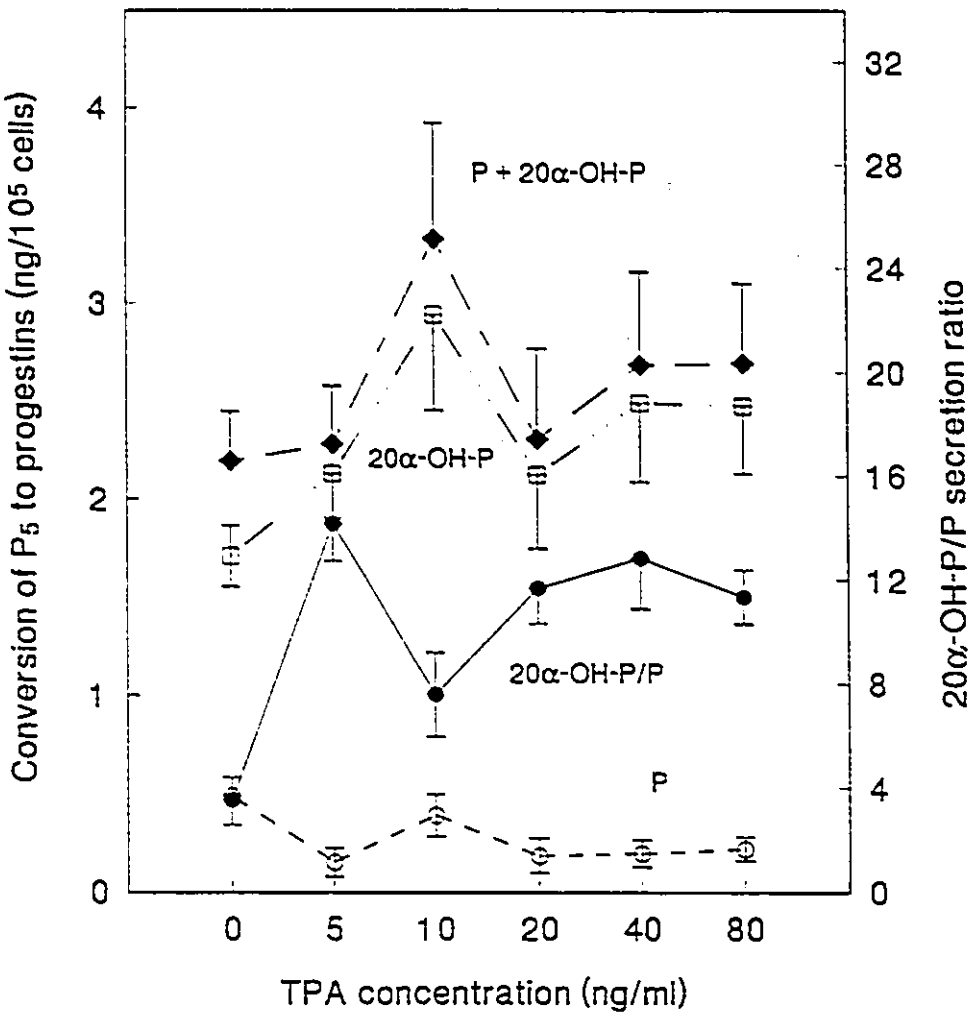

Figure 21 Concentration-dependent influence of TPA on the conversion of exogenous P_5 (0.1 µM) to P, 20α–OH-P and P+20α–OH-P (in the presence of 0.75 mM AGP) during a 24 h incubation period. Values represent means ±SEM of 10 incubations from 3 experiments.

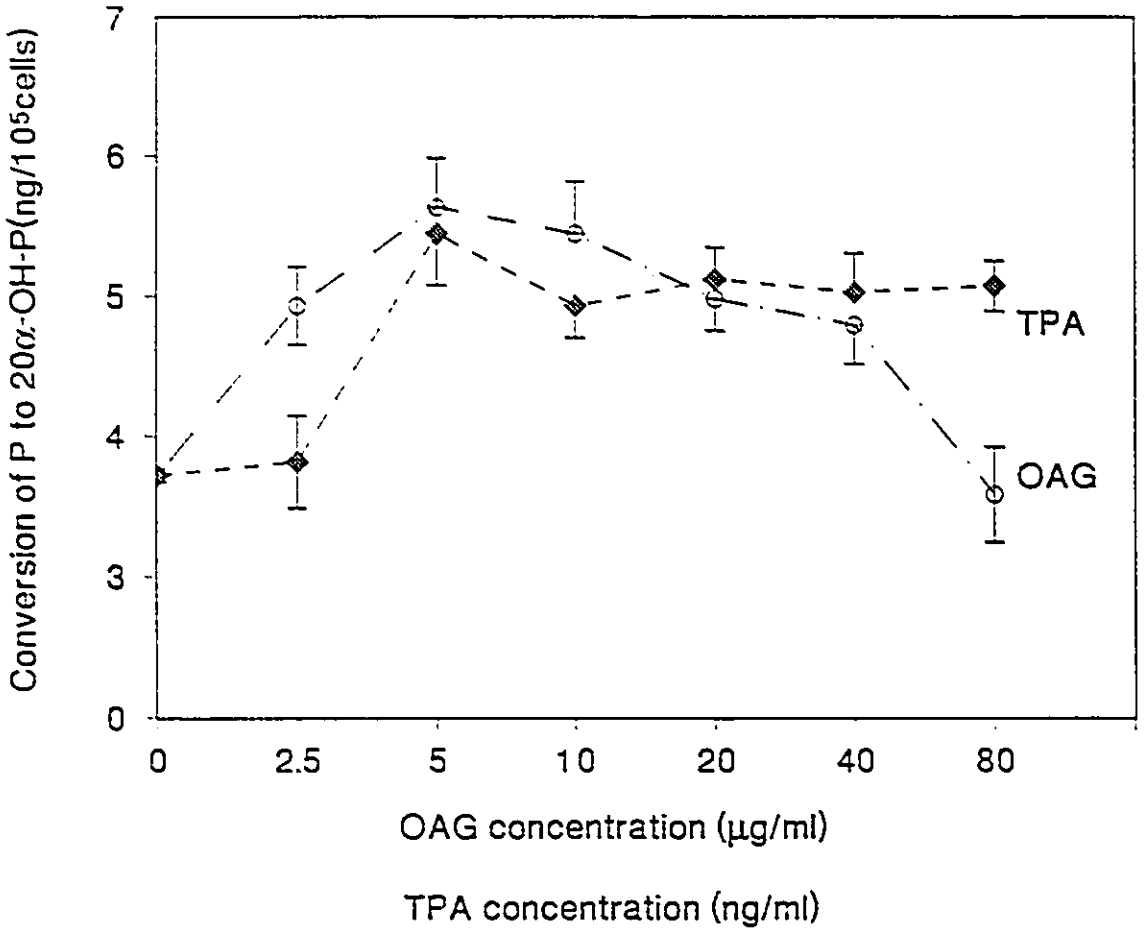

Figure 22 Concentration-dependent influence of TPA and OAG on the conversion of exogenous P (0.1 µM) to 20α-OH-P (in the presence of 25 µM of cyanoketone) in 24 h cultures. Values represent means ±SEM of 9 incubations from 3 experiments.

II. IS PKC INVOLVED IN THE STIMULATION OF GRANULOSA CELL STEROIDOGENESIS BY FSH? DOES IT PARTICIPATE IN THE MODULATION OF FSH-INDUCED STEROID HORMONE PRODUCTION?

A. Influence of FSH on Basal Secretion of P_5, P and 20α-OH-P

FSH significantly ($p<0.05$) increased granulosa cell secretion of P_5 (measured in the presence of cyanoketone), P and 20α-OH-P (Table 8). The effect of the gonadotropin on total progestin (P+20α-OH-P) secretion was concentration-dependent and maximal stimulation was noted at 150 ng/ml (Fig. 23). Time-course studies on steroid secretion by granulosa cell revealed that maximal stimulation by FSH was evident during the first 6 h of culture (Figs. 24 and 25). In the first 3 h, FSH (150 ng/ml) stimulated the secretion of P_5 (in the presence of cyanoketone), P, 20α-OH-P and P+20α-OH-P by about 6-(Fig. 24), 8-, 6- and 7- fold (Fig. 25), respectively. In most cultures, no further increase in steroids secretion was noted after 12 h of incubation with FSH, although the secretion of the steroids by granulosa cells in the absence of the gonadotropin increased with duration of incubation (Figs. 24 and 25).

Table 8 Influence of FSH (150 ng/ml), OAG (20 μg/ml) and TPA (40 ng/ml) on P_5, P and 20α-OH-P secretion during a 6 and 24 h incubation period.

Treatment Groups	Steroid secretion (ng/10^5 cells)					
	6 h incubation			24 h incubation		
	P_5	P	20α - OH-P	P_5	P	20α - OH-P
Control	0.60 ± 0.03	0.22 ± 0.02	0.43 ± 0.04	1.37 ± 0.22	0.35 ± 0.03	1.15 ± 0.22
FSH (150ng/ml)	2.21 ± 0.22 [a]	1.46 ± 0.14 [a]	3.17 ± 0.25 [a]	2.89 ± 0.34 [a]	0.91 ± 0.05 [a]	2.81 ± 0.20 [a]
OAG (20 μg/ml)	1.69 ± 0.24 [a]	0.29 ± 0.03	1.17 ± 0.10 [a]	2.55 ± 0.04 [a]	0.54 ± 0.04 [a]	2.43 ± 0.03 [a]
TPA (40ng/ml)	0.87 ± 0.08	0.28 ± 0.02	0.64 ± 0.07 [a]	0.81 ± 0.07 [a]	0.23 ± 0.02 [a]	1.37 ± 0.14

Values represent mean ± SEM of 18 incubations from 6 experiments (P and 20α - OH-P secretion) or 6 incubations from 2 experiments (P_5 secretion). P_5 secretion was assessed in the presence of 25 μM of cyanoketone (to inhibit further metabolism of P_5).

[a] - $p < 0.05$ (compared to control)

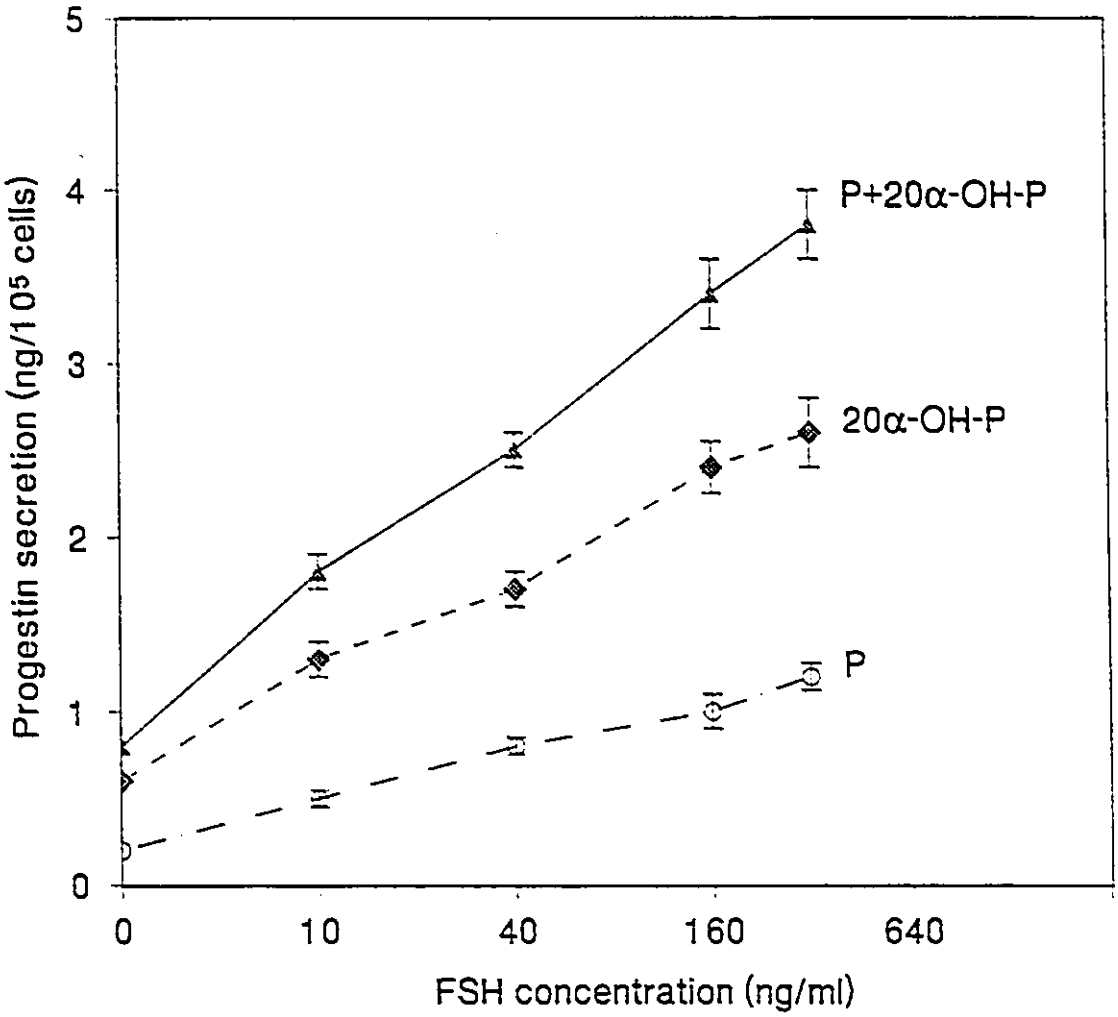

Figure 23 Dose-dependent effect of FSH on the secretion of P, 20α-OH-P and P+20α-OH-P in 24 h cultures. Values represent means ±SEM of 6 incubations from 2 experiments.

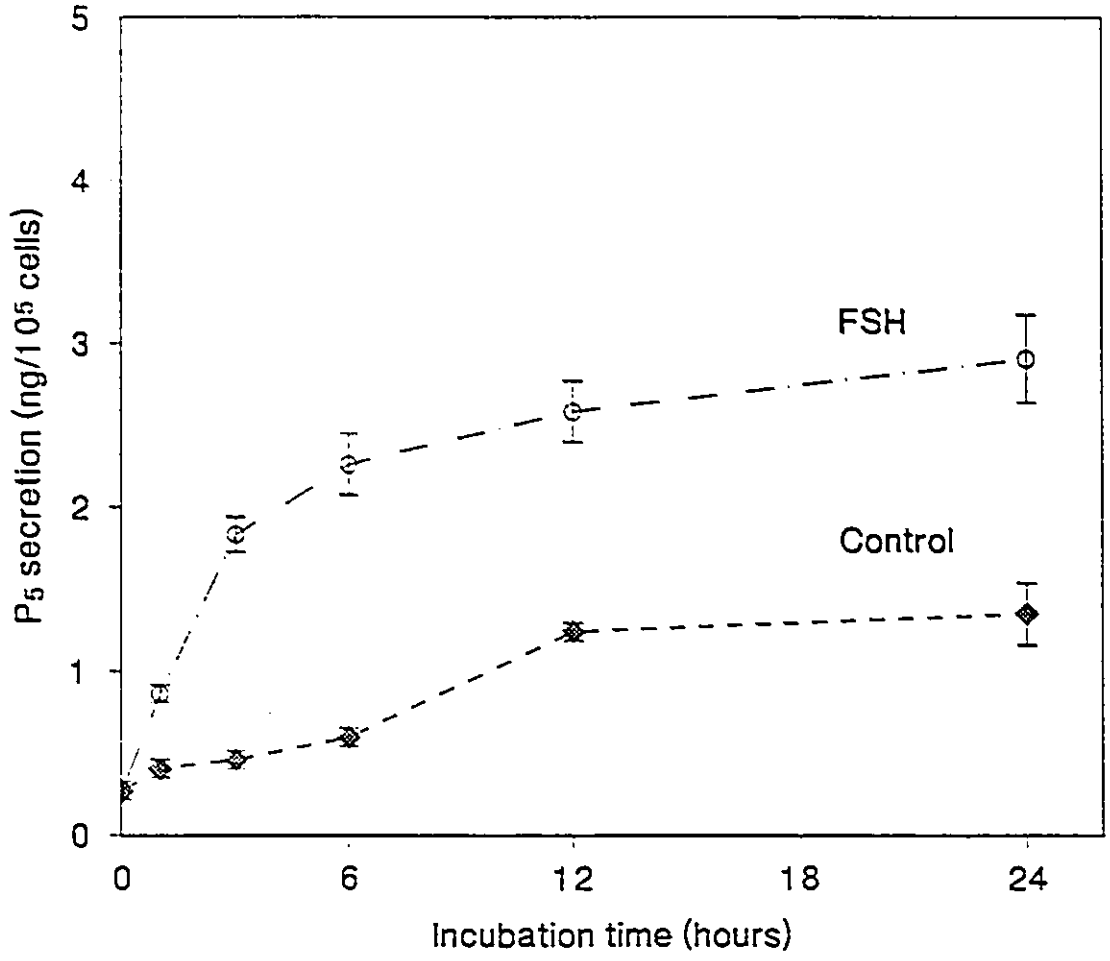

Figure 24 Time course of the effect of FSH (150 ng/ml) on granulosa cell P_5 secretion in the presence of 25 μM cyanoketone. Values represent means ±SEM of 6 incubations from 2 experiments.

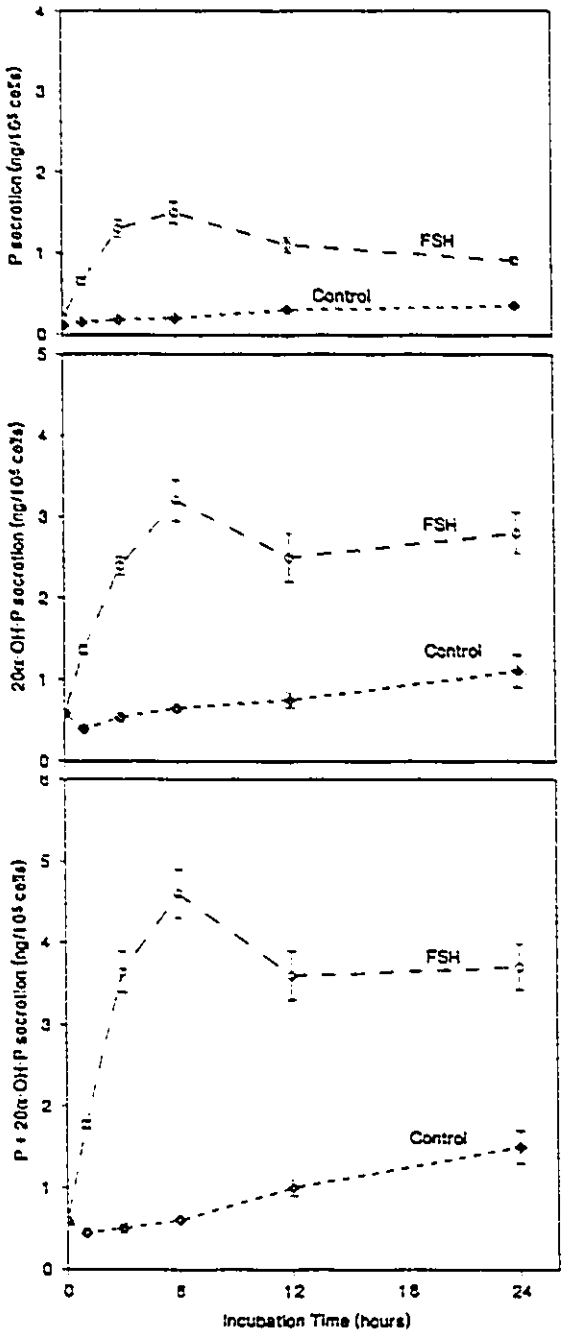

Figure 25 Time course of the effect of FSH (150 ng/ml) on secretion of P, 20α-OH-P and P+20α-OH-P. Values represent means ±SEM of 18 incubations from 6 experiments.

B. Possible Involvement of PKC in the Stimulation of Granulosa Cell Steroidogenesis by FSH

1) Effect of 20 h Pretreatment with TPA on FSH-Stimulated P and 20α-OH-P Secretion

Granulosa cells were first cultured for 20 h with TPA (40ng/ml) and subsequently for an additional 6 h in fresh medium containing FSH (150ng/ml). Pretreatment of granulosa cells with the phorbol ester resulted in a significant (~40%; $p<0.05$) suppression of the FSH-stimulated secretion of P+20α-OH-P (Fig. 26).

2) Influence of PKC Inhibitors on FSH-, $(Bu)_2$cAMP- and Forskolin-Stimulated Steroidogenesis

Granulosa cells were incubated for 24 h in the presence of FSH (150 ng/ml), $(Bu)_2$cAMP (1.5 mM) or forskolin (100 μM) and either H_7 or ESP. The inhibitors were added to the cells 1 hour before FSH. H_7 (100 μM) and ESP (10 μM) significantly (30-40 %; $p<0.05$) inhibited FSH-stimulated P+20α-OH-P secretion (Table 9). However, ESP (1-10 μM) did not affect $(Bu)_2$cAMP- or forskolin-stimulated steroidogenesis (Table 9). Higher concentrations of the inhibitors (1 mM of H_7 and 100 μM of ESP) significantly ($p<0.05$) reduced cell viability.

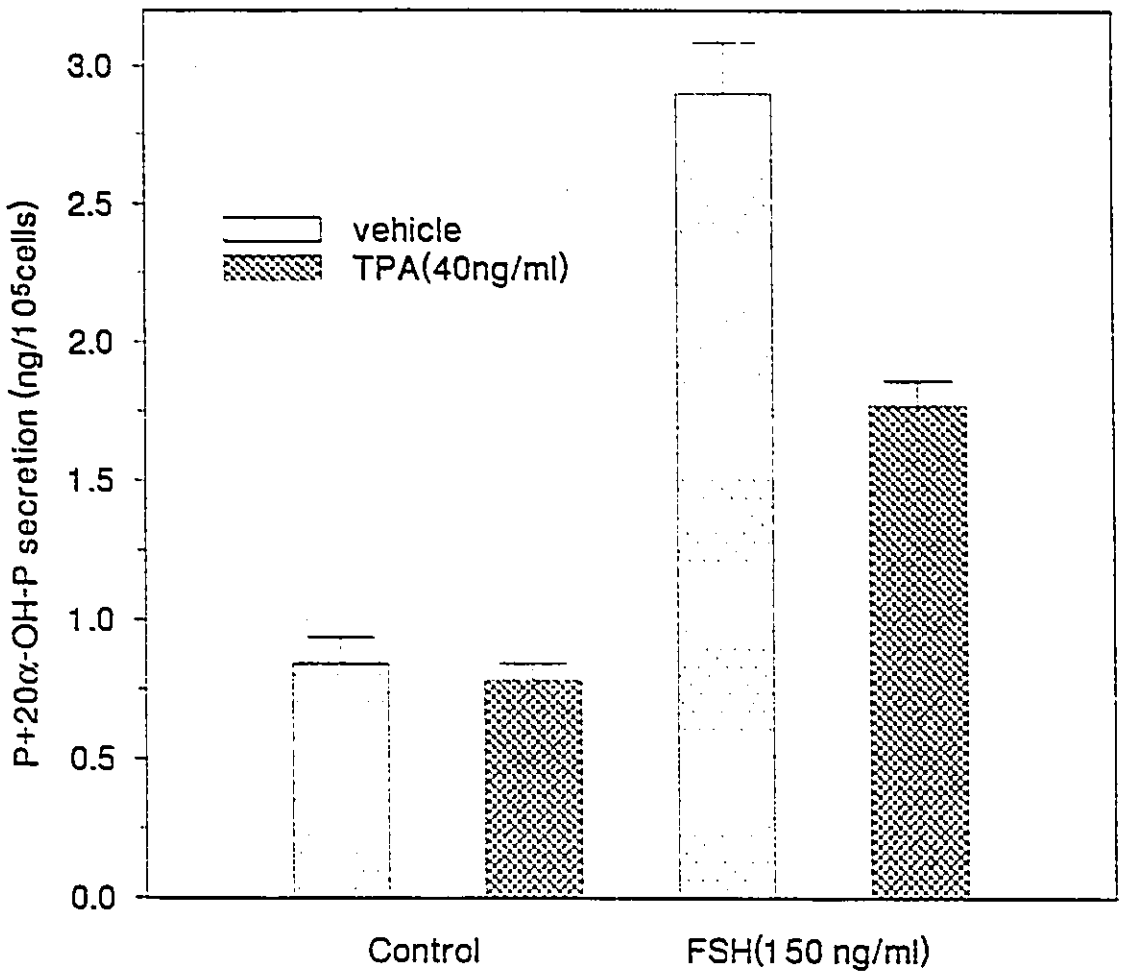

Figure 26 Effect of 20 h pretreatment with TPA (40 ng/ml) on FSH (150 ng/ml) - stimulated secretion of total progestins (P+20α-OH-P) during a 6 h incubation period. Values represent means ±SEM of 9 incubations from 3 experiments.

Table 9 Influence of various concentrations of PKC inhibitors (H-7 and ESP) on FSH (150 ng/ml)-, $(Bu)_2$cAMP (1.5 mM)- and Forskolin (100μM)-stimulated total progestin (P+20α-OH-P) secretion in 24 h incubations.

Treatment Groups	P+20α-OH-P (ng/10^5 cells)
Control	0.75 ± 0.08
FSH (150 ng/ml)	3.34 ± 0.23 [a]
FSH (150 ng/ml) + H_7 (10μM)	2.94 ± 0.24 [a]
FSH (150 ng/ml) + H_7 (100μM)	2.11 ± 0.10 [a b]
FSH (150 ng/ml) + ESP (1μM)	3.01 ± 0.11 [a]
FSH (150 ng/ml) + ESP (10μM)	1.99 ± 0.12 [a b]
$(Bu)_2$cAMP (1.5 mM)	10.81 ± 0.62 [a]
$(Bu)_2$cAMP (1.5 mM)+ESP(1μM)	11.93 ± 0.95 [a]
$(Bu)_2$cAMP (1.5 mM)+ESP(10μM)	8.66 ± 0.70 [a]
Forskolin (100μM)	12.64 ± 0.93 [a]
Forskolin (100μM)+ESP(1μM)	10.72 ± 0.75 [a]
Forskolin (100μM)+ESP(10μM)	9.16 ± 0.87 [a]

Results represent means ± SEM of 6 incubations from 2 experiments.

[a] - $p<0.05$ (compared to control)

[b] - $p<0.05$ (compared to FSH alone)

C. Possible Involvement of PKC in the Modulation of FSH-Induced Granulosa Cell Steroidogenesis.

1) Influence of OAG and TPA on FSH-Stimulated P_5 Secretion

Granulosa cells were incubated for up to 24 h with FSH (150 ng/ml) and 25 μM of cyanoketone in the presence or absence of OAG (20 μg/ml) or TPA (40 ng/ml). FSH (150 ng/ml) significantly stimulated synthesis of P_5 from endogenous cholesterol (in the presence of cyanoketone) already in the first hour incubation but maximal response was observed during the 3-6 h of incubation (Fig. 24). OAG and TPA exerted opposite effects on FSH-stimulated P_5 accumulation (Fig. 27). Whereas secretion of the steroid by the granulosa cells in response to FSH was markedly enhanced by OAG (2-fold with 20 μg/ml; $p<0.05$) throughout the incubation period (1-24 h), it was significantly inhibited (50%) by TPA (40ng/ml) from 6 to 24 h of incubation ($p<0.05$).

2) Influence of OAG and TPA on FSH-Stimulated Progestin Secretion

Granulosa cells were cultured for 0-24 h with FSH (150 ng/ml) in the presence or absence of OAG (20 μg/ml) or TPA (40ng/ml). FSH (150 ng/ml) markedly and concentration-dependently increased total progestin (P+20α-OH-P) secretion by granulosa cells in vitro (Fig. 25). OAG and TPA exerted divergent effects on FSH-stimulated progestin secretion (Figs 28 and 29). While OAG (20 μg/ml) had no apparent influence on P secretion up to 12 h of incubation in the presence of the gonadotropin, it significantly ($p<0.05$) stimulated 20α-OH-P and total progestin (P+20α-OH-P) secretion during all incubation periods (Fig. 28). The action of OAG was concentration-dependent and maximal stimulation (60%) was noted with 20 μg/ml at 24 h of culture (Fig. 29).

P+20α-OH-P secretion at all concentrations of FSH examined (9.4-150 ng/ml) was dose-dependently enhanced by OAG ($p<0.05$; Fig. 30). In contrast, TPA attenuated FSH-stimulated P, 20α-OH-P and consequently total progestin (P+20α-OH-P) secretion

in a concentration-dependent manner, with maximal suppression (50%) of the gonadotropic response observable at 40 ng/ml (Figs 28 and 29). The effect of the PKC activator on steroid secretion was significant ($p<0.05$) throughout the incubation period (Fig. 28). The enhancement of the FSH- induced steroidogenesis by OAG appeared to be inversely proportional to the response of the cells to the gonadotropin: the greater the FSH stimulation, the smaller was the OAG effect on progestin secretion (Table 10). Maximal stimulation by FSH (group III) was not further potentiated by OAG. In contrast, the inhibitory action of TPA did not appear to be influenced by the responsiveness of the cells to FSH (Table 10).

3) Effects of DiC_8, Diolein, 4α-Phorbol 12,13-Didecanoate and Phorbol-13-Monoacetate on FSH-Stimulated Progestin Secretion.

To investigate whether the actions of OAG and TPA on FSH-stimulated progestin secretion were specific, granulosa cells were incubated also with two additional diacylglycerols (DiC_8 and diolein) and two phorbol esters with no tumor promoting activity (4α-phorbol 12,13-didecanoate and phorbol-13-monoacetate). Like OAG (Fig. 29), DiC_8 (5-80 μg/ml) significantly ($p<0.05$) and dose-dependently potentiated the stimulatory action of FSH (150 ng/ml) on the secretion of P+20α-OH-P during the 24 h culture period (Fig. 31). At the maximal stimulatory concentration (20 μg/ml), DiC_8 increased progestin secretion by 2-fold. In contrast to permeable diacylglycerols, the nonpermanent diolein failed to influence gonadotropin-stimulated steroidogenesis in vitro (Fig. 32). Likewise, 4α phorbol 12,13-didecanoate and phorbol-13-monoacetate (40 ng/ml) had no effect on FSH-stimulated P and 20α-OH-P secretion during either 6 or 24 h of culture (Table 11).

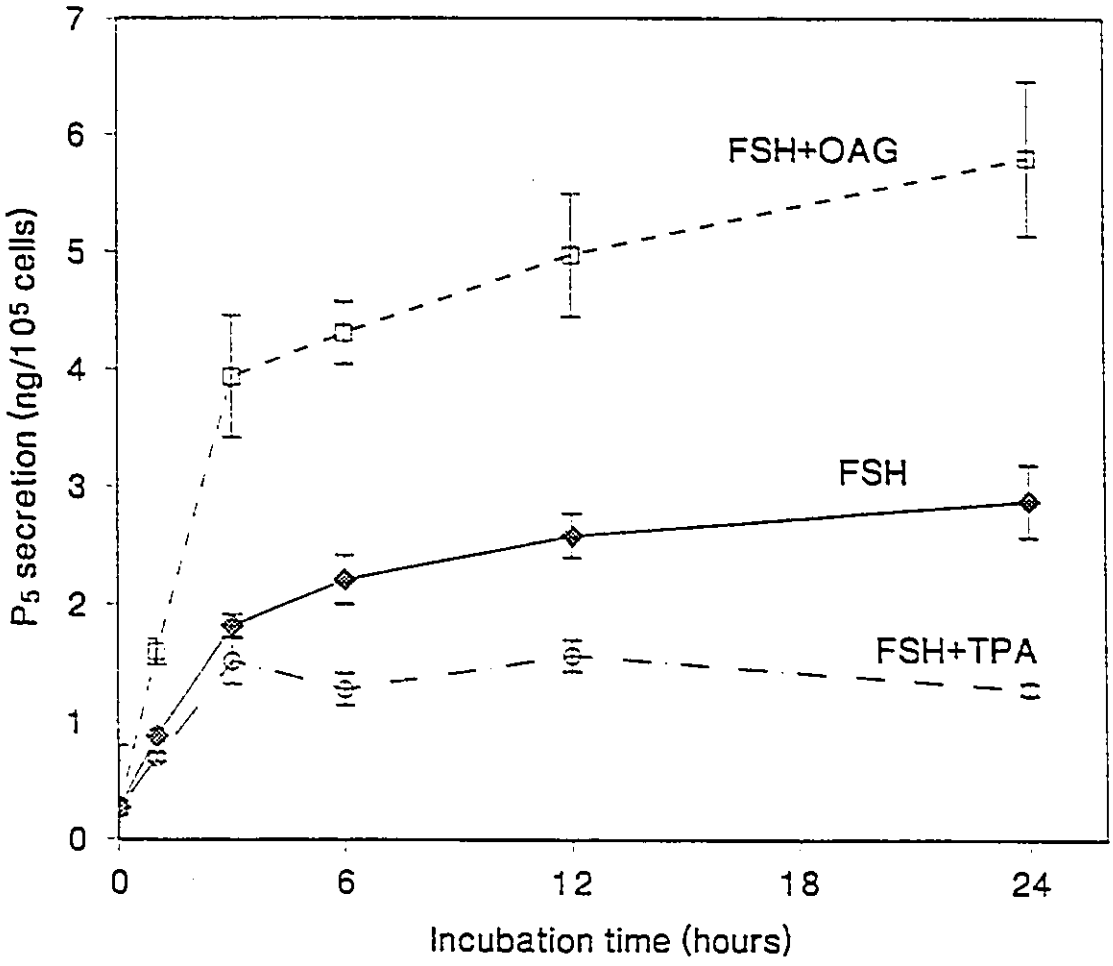

Figure 27 Time course of the effects of OAG (20 µg/ml) and TPA (40 ng/ml) on FSH (150 ng/ml) - stimulated granulosa cell P_5 secretion in the presence of 25 µM of cyanoketone. Values represent means ±SEM of 6 incubations from 2 experiments.

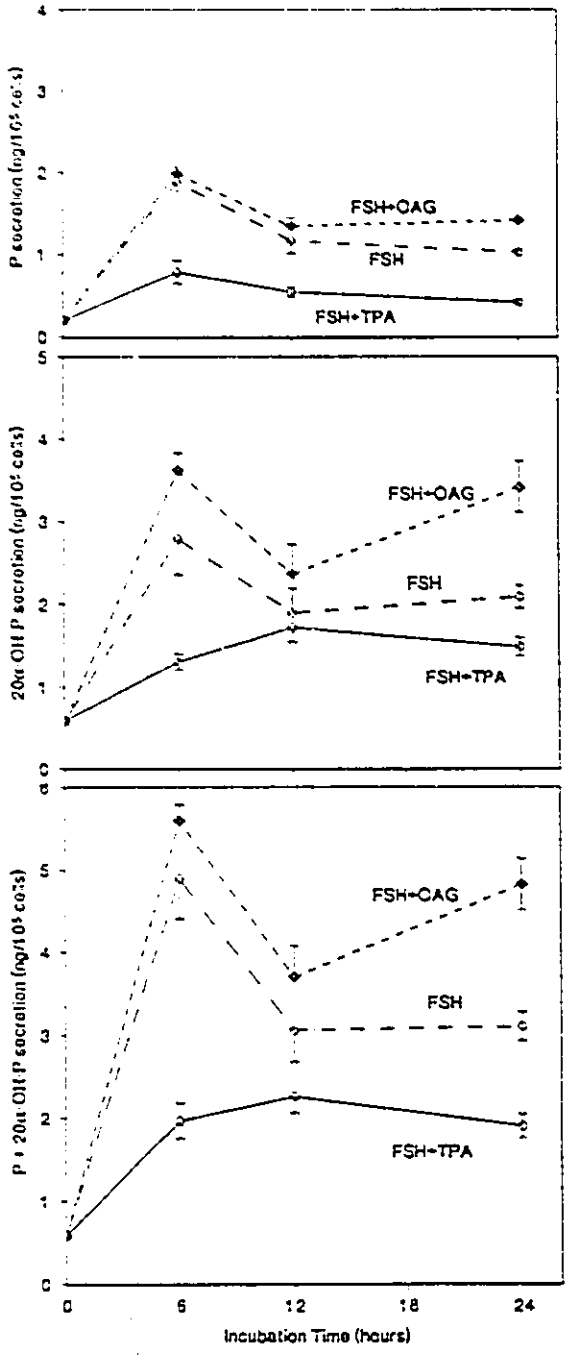

Figure 28 Time course of the effects of OAG and TPA on FSH (150 ng/ml) - stimulated secretion of P and 20α-OH-P . Values represent means ±SEM of 9 incubations from 3 experiments.

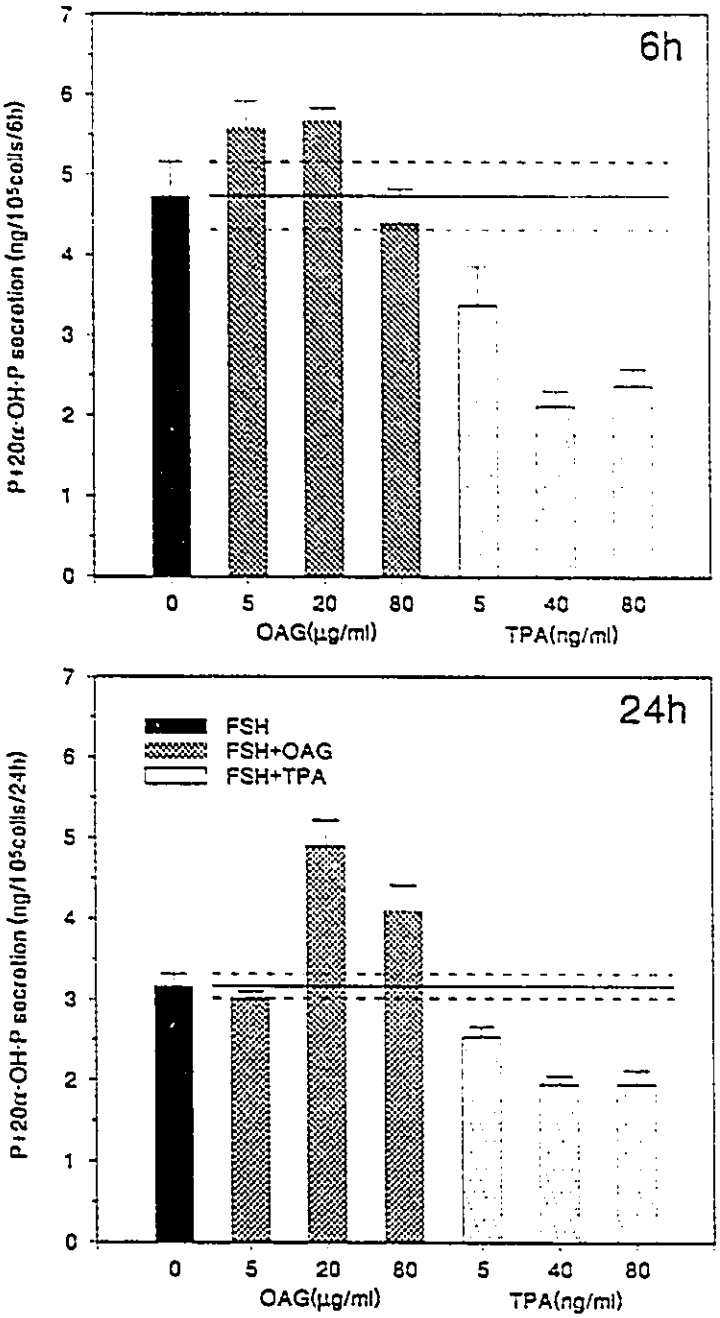

Figure 29 Concentration-dependent effects of OAG and TPA on FSH (150 ng/ml) - stimulated total progestin (P+20α-OH-P) secretion by granulosa cells during a 6 h and 24 h incubation period. Values represent means ±SEM of 9 incubations from 3 experiments.

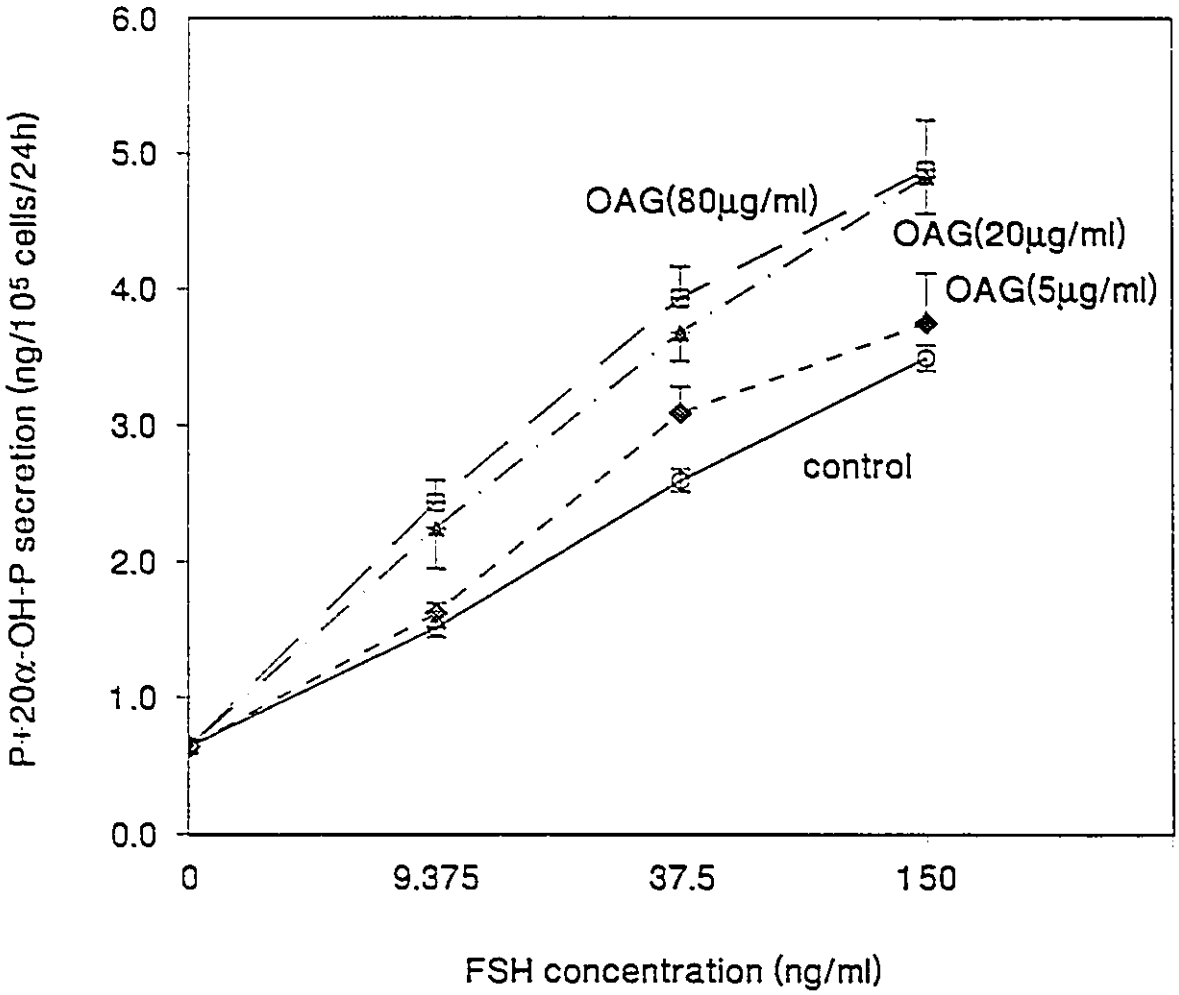

Figure 30 Dose-dependent effect of OAG on total progestin (P+20α-OH-P) secretion induced by various concentrations of FSH during a 24 h culture period. Values represent means ±SEM of 9 incubations from 3 experiments.

Table 10 Relationship between the magnitude of FSH (150 ng/ml) stimulation of total progestin (P+20α - OH-P) secretion in granulosa cells and their responsiveness to OAG (20 μg/ml) or TPA (40 ng/ml).

Treatment Groups	P + 20 α -OH - P secretion (ng/10^5 cells)		
	I	II	III
Control	0.94±0.07	1.11±0.15	1.08±0.10
FSH (150 ng/ml)	2.30±0.28[a]	4.42±0.21[a]	5.13±0.30[a]
FSH (150 ng/ml) + OAG (20μg/ml)	4.05±0.18[ab]	5.60±0.45[ab]	5.46±0.37[a]
FSH (150 ng/ml) + TPA (40 ng/ml)	1.82±0.13[ab]	2.31±0.13[ab]	2.60±0.28[ab]

I. II. III - groups with different magnitude of response to FSH stimulation.

Values represent means ± SEM of 12 incubations from 4 experiments.

a $p < 0.05$ (compared to control)

b $p < 0.05$ (compared to FSH alone).

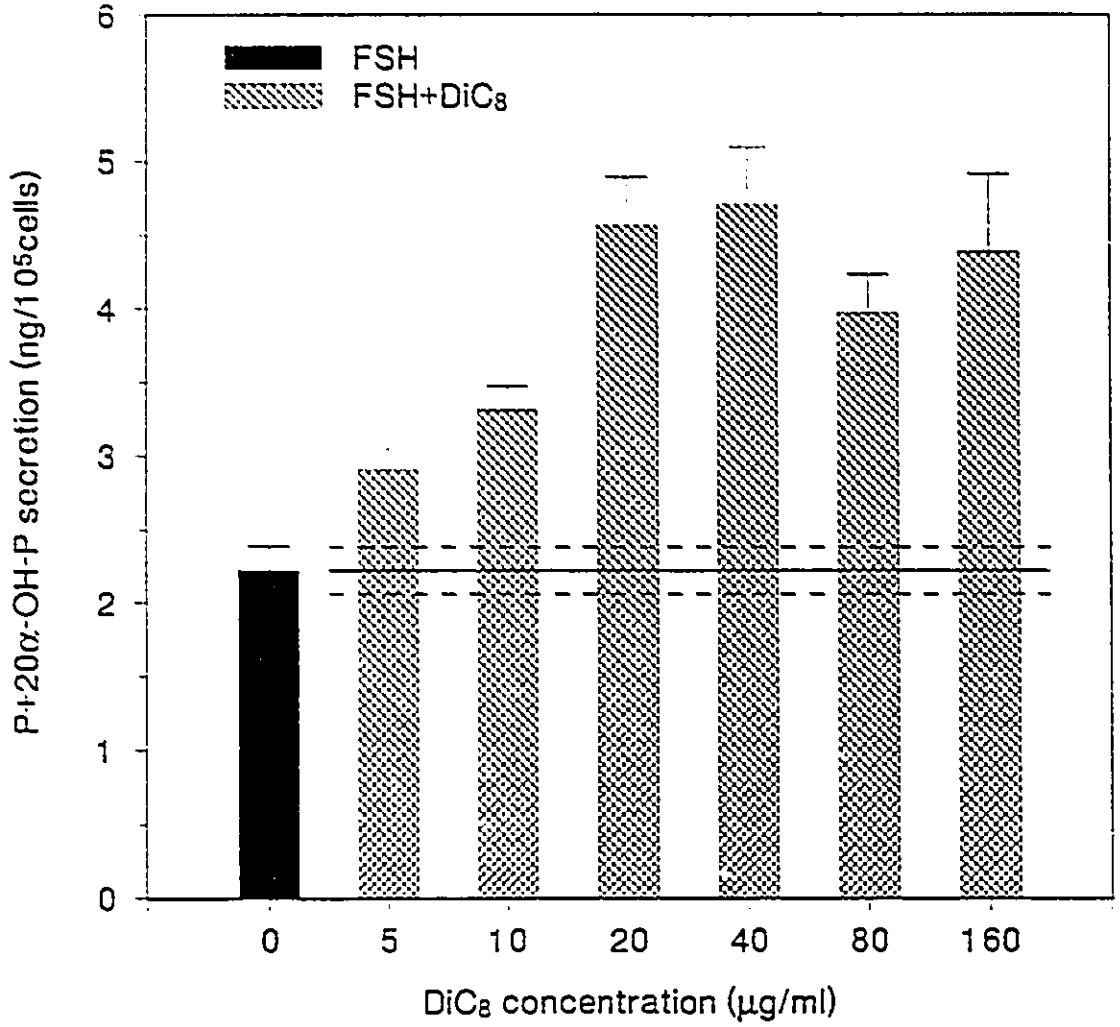

Figure 31 Concentration-dependent influence of DiC_8 on FSH (150 ng/ml)-stimulated total progestin (P+20α-OH-P) secretion during a 24 h incubation period. Values represent means ±SEM of 9 incubations from 3 experiments.

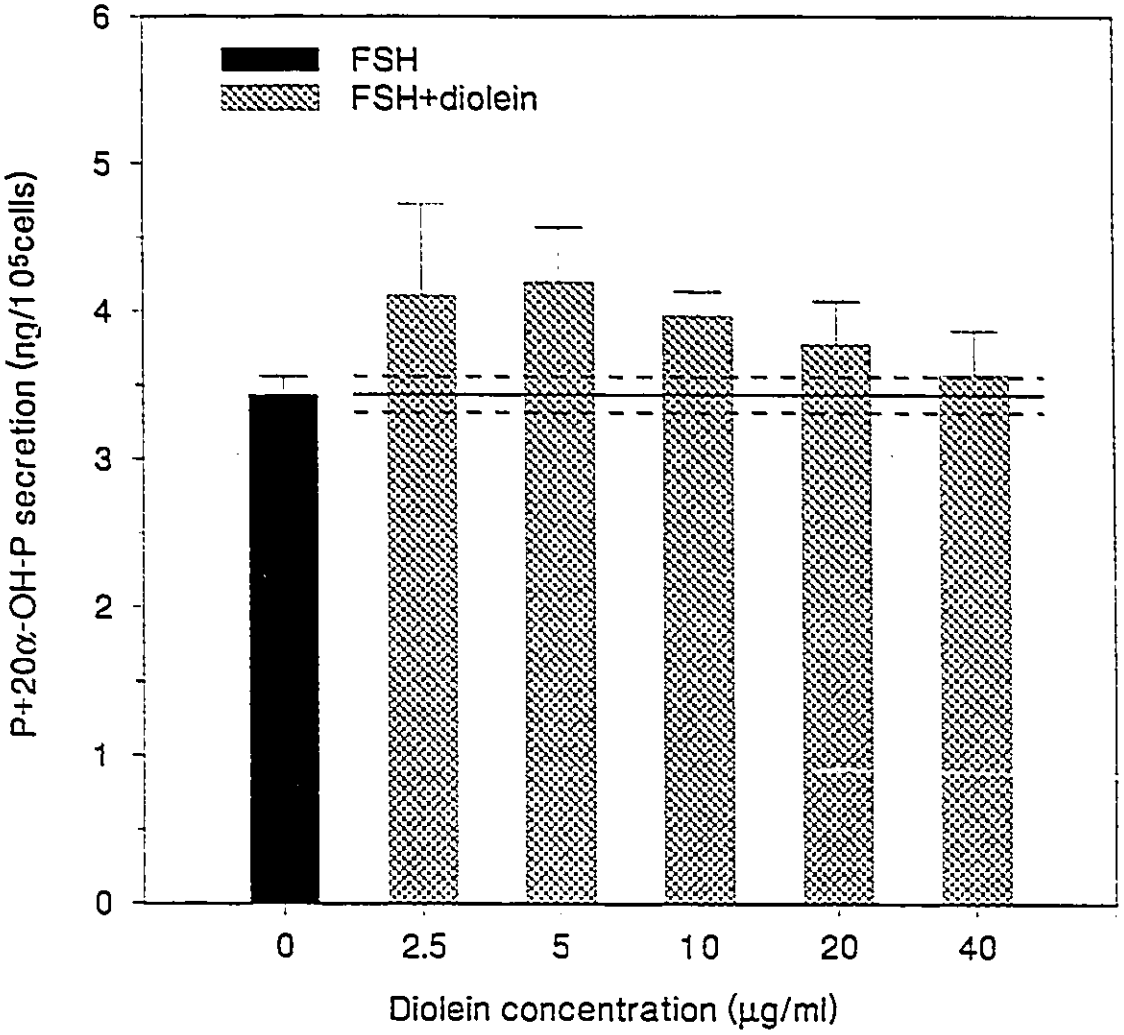

Figure 32 Lack of effect of diolein on FSH (150 ng/ml)-stimulated total progestin (P+20α-OH-P) secretion in 24 h cultures. Values represent means ±SEM of 6 incubations from 2 experiments.

Table 11 Influence of 4α-phorbol 12,13-didecanoate (40 ng/ml) and phorbol-13-monoacetate (40 ng/ml) on FSH (150 ng/ml)-stimulated total progestin (P+20α-OH-P) secretion in 6 and 24 h cultures.

	P + 20α - OH-P secretion (ng/10^5 cells)	
Treatment Groups	6 h	24 h
Control	0.62 ± 0.06	1.20 ± 0.21
FSH (150 ng/ml)	4.75 ± 0.31[a]	4.32 ± 0.24[a]
FSH (150ng/ml + phorbol-13-monoacetate (40 ng/ml)	5.06 ± 0.48[a]	3.86 ± 0.35[a]
FSH (150 ng/ml) + 4α-phorbol 12,13-didecanoate (40 ng/ml)	4.59 ± 0.56[a]	4.11 ± 0.50[a]

Values represent means ± SEM of 6 replicate incubations from 2 experiments.

[a] - $p < 0.05$ (compared to control)

D. If PKC is Involved in the Regulation of FSH-Stimulated Steroidogenesis, Does it Exert its Effect Through the Modulation of the cAMP Signalling Pathway?

FSH is believed to increase granulosa cell steroidogenesis by stimulating, at least in part the adenylyl cyclase-cAMP second messenger system. Since diacylglycerols and tumor promoting phorbol ester are known to affect FSH-induced biosynthesis of steroid hormones (Welsh et al 1984; Kawai and Clark 1985; Shinohara et al 1985, 1986; Veldhuis and Demers 1986) it is possible that they exert their influence by modulating the action of cAMP produced in response to FSH.

1) Time and Dose-Dependent Effects of OAG, DiC_8, Diolein and TPA on $(Bu)_2$ cAMP-Stimulated Steroid Secretion

To explore the possibility of an involvement of PKC in the regulation of cAMP-induced steroidogenesis, granulosa cells were incubated for different durations with OAG, DiC_8, diolein or TPA in the absence or presence of the cAMP analogue $(Bu)_2$ cAMP (Fig. 33). $(Bu)_2$ cAMP (1.5 mM) markedly increased ($p<0.05$) granulosa cell secretion of P_5 (approximately 6-fold; Table 12) and total progestins (P+20α-OH-P; 15-fold; Fig. 34) during a 24 h culture period. The effect of the cAMP analogue was time-dependent as evident by a larger stimulation with an increased duration of incubation (Fig. 34). OAG (5-20 µg/ml) significantly ($p<0.05$) and dose-dependently enhanced $(Bu)_2$cAMP-stimulated P+20α-OH-P secretion (Fig. 35), although, its effect was evident only during 24 h cultures (Figs. 34-35). DiC_8 (5-20 µg/ml) also potentiated the $(Bu)_2$cAMP-induced secretion of total progestins (in a concentration-dependent manner) at 24 h, whereas diolein (2.5-80ng/ml) was ineffective (Table 13). Maximal stimulation by DiC_8 (~60%) was observed at 10 µg/ml (Fig. 36).

In contrast, TPA significantly inhibited $(Bu)_2$cAMP-stimulated P_5 (Table 12) and P+20α-OH-P secretion (Figs 34 and 35). This effect was time- and concentration-dependent, with maximal inhibition (40% - 50%) noted at 5 ng/ml during a 24 h culture period (Figs 34 and 35)

2) Concentration-Dependent Effects of OAG and TPA on Forskolin-Stimulated P+20α-OH-P Secretion

To determine whether the effects of OAG and TPA on FSH-induced steroid secretion could be due to their influence on the coupling of FSH receptor to the adenylyl cyclase, granulosa cells were cultured with OAG or TPA in the presence or absence of various concentrations of forskolin, a direct activator of the enzyme. In 24 h cultures, forskolin (1-100 μM) dose-dependently increased total progestin (P+20α-OH-P) secretion ($p<0.05$), maximal stimulation (10-fold) was observed at 50 μM (Fig. 37). Whereas OAG (20 μg/ml) significantly ($p<0.05$) potentiated the stimulatory effect of forskolin (observed at concentrations 10-100 μM) (Fig 37), the influence of TPA (40 and 80 ng/ml) was biphasic (stimulatory at 10 μM and inhibitory at 50-100 μM of forskolin; Fig. 37).

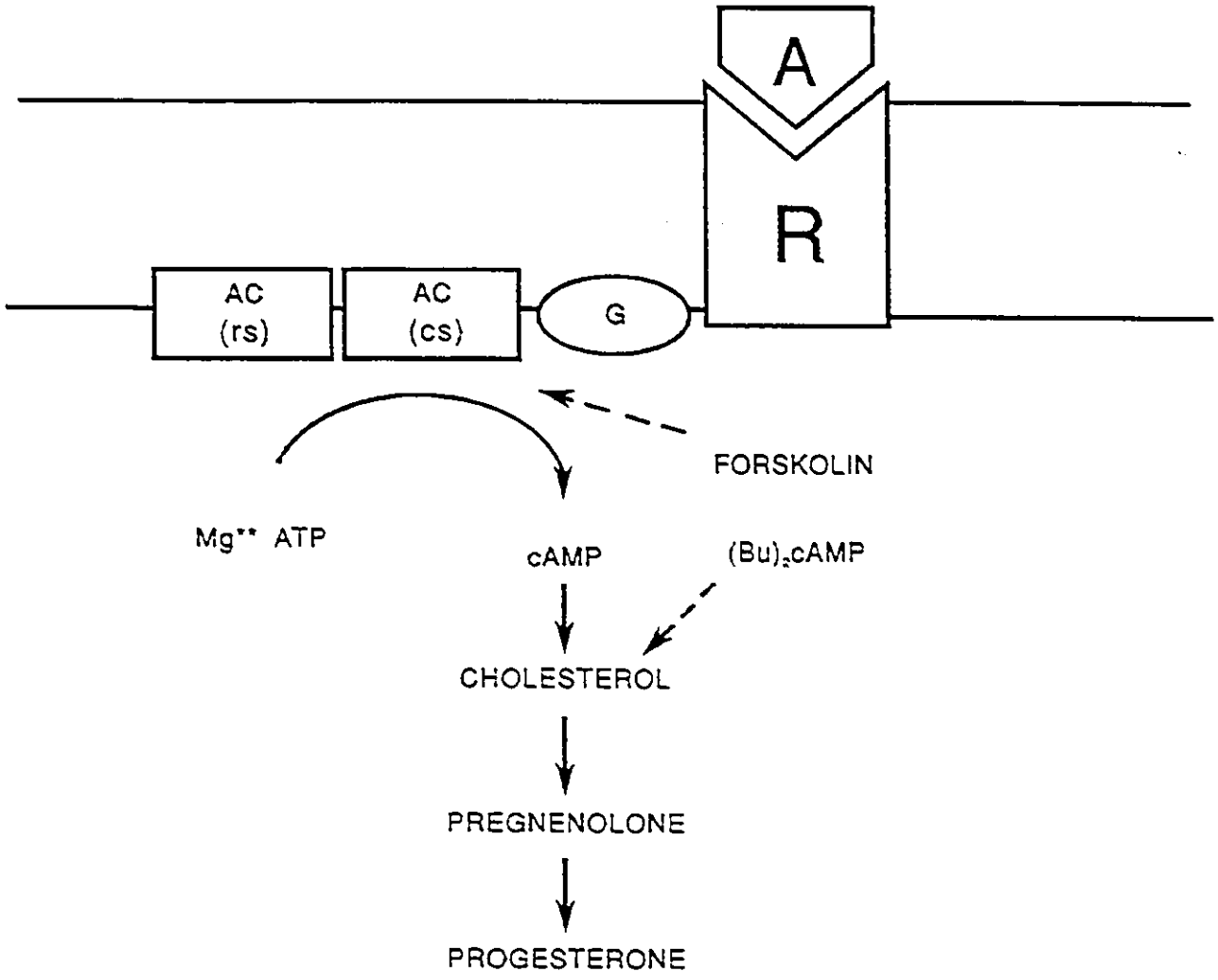

Figure 33 Sites of action of forskolin and $(Bu)_2$cAMP on granulosa cell steroidogenesis.

G	- G protein	AC	- adenylyl cyclase
A	- agonist	R	- receptor
rs	- regulatory subunit of AC	cs	- catalytic subunit of AC

Table 12 Effect of TPA (40 ng/ml) on basal and $(Bu)_2$cAMP (1.5 mM)-stimulated P_5 secretion (in the presence of 25µM of cyanoketone) in 24 h cultures.

Treatment Groups	P_5 secretion (ng/10^5 cells)
Control	0.32 ± 0.03
$(Bu)_2$cAMP(1.5mM)	1.93 ± 0.15 [a]
TPA(40ng/ml)	0.50 ± 0.04
$(Bu)_2$cAMP(1.5mM)+TPA(40ng/ml)	1.04 ± 0.11 [a b]

Values represent means ± SEM of 7 incubations from 2 experiments.

[a] - $p<0.05$ (compared to control)

[b] - $p<0.05$ (compared to $(Bu)_2$cAMP alone)

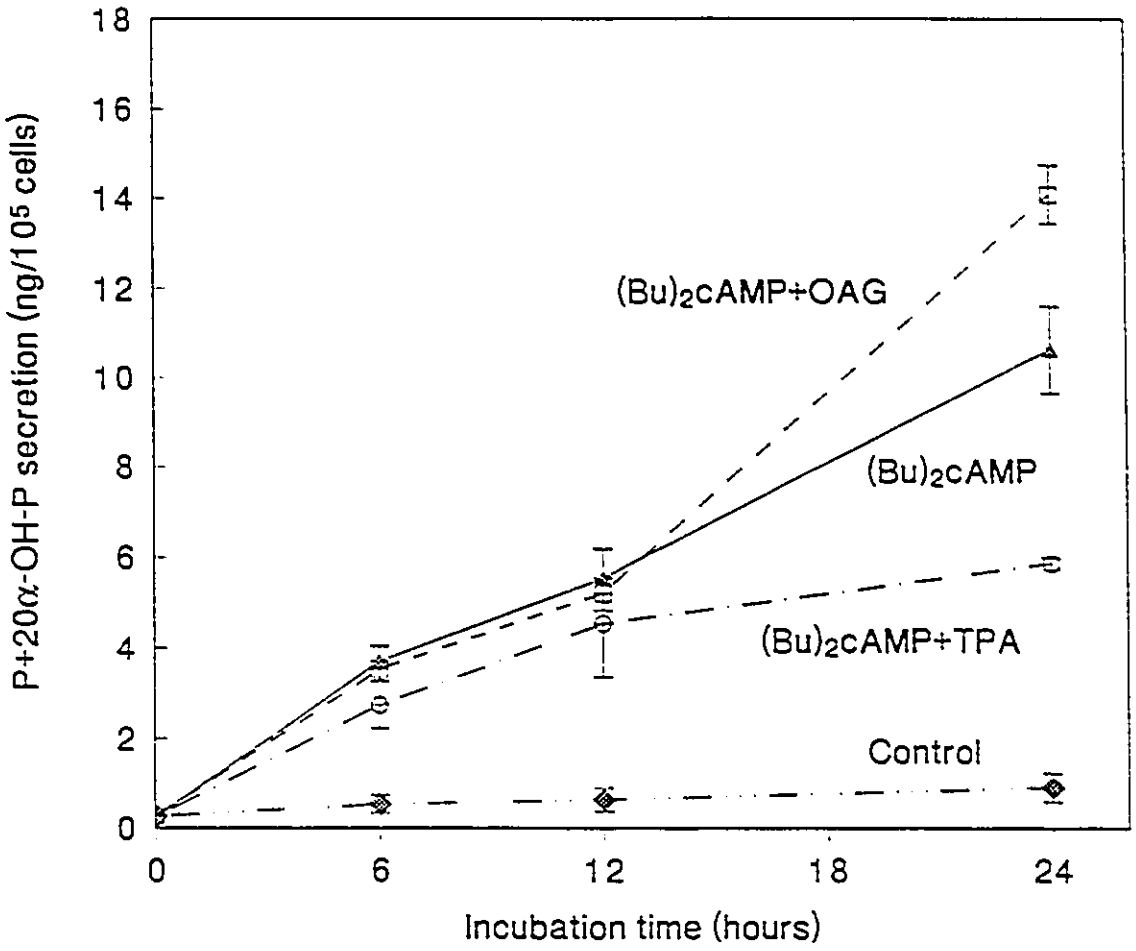

Figure 34 Time course of the effect of OAG (20 µg/ml) and TPA (40 ng/ml) on $(Bu)_2$cAMP (1.5 mM)-stimulated secretion of total progestins (P+20α-OH-P). Values represent means ±SEM of 6 incubations from 2 experiments.

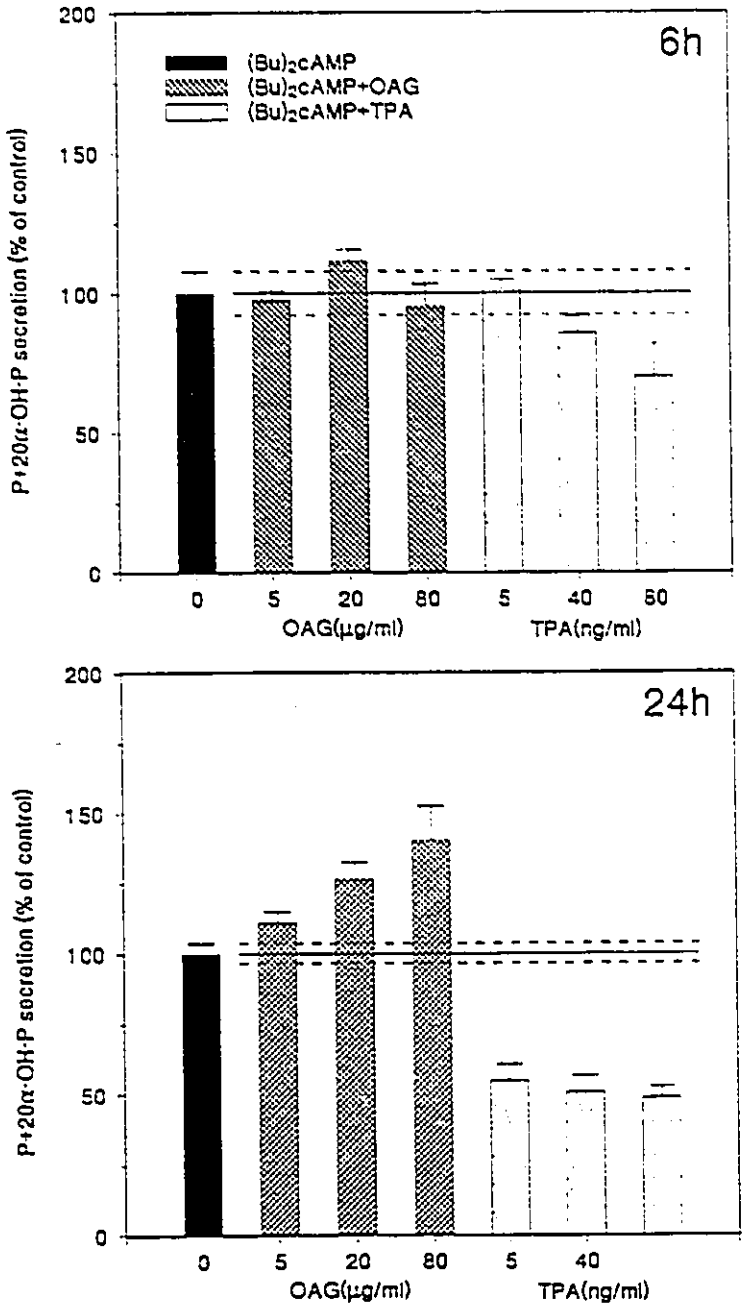

Figure 35 Concentration-dependent influence of OAG and TPA on $(Bu)_2$ cAMP (1.5mM)-stimulated secretion of total progestins (P+20α-OH-P) during 6 h and 24 h incubations. Values represent means ±SEM of 9 incubations from 3 experiments.

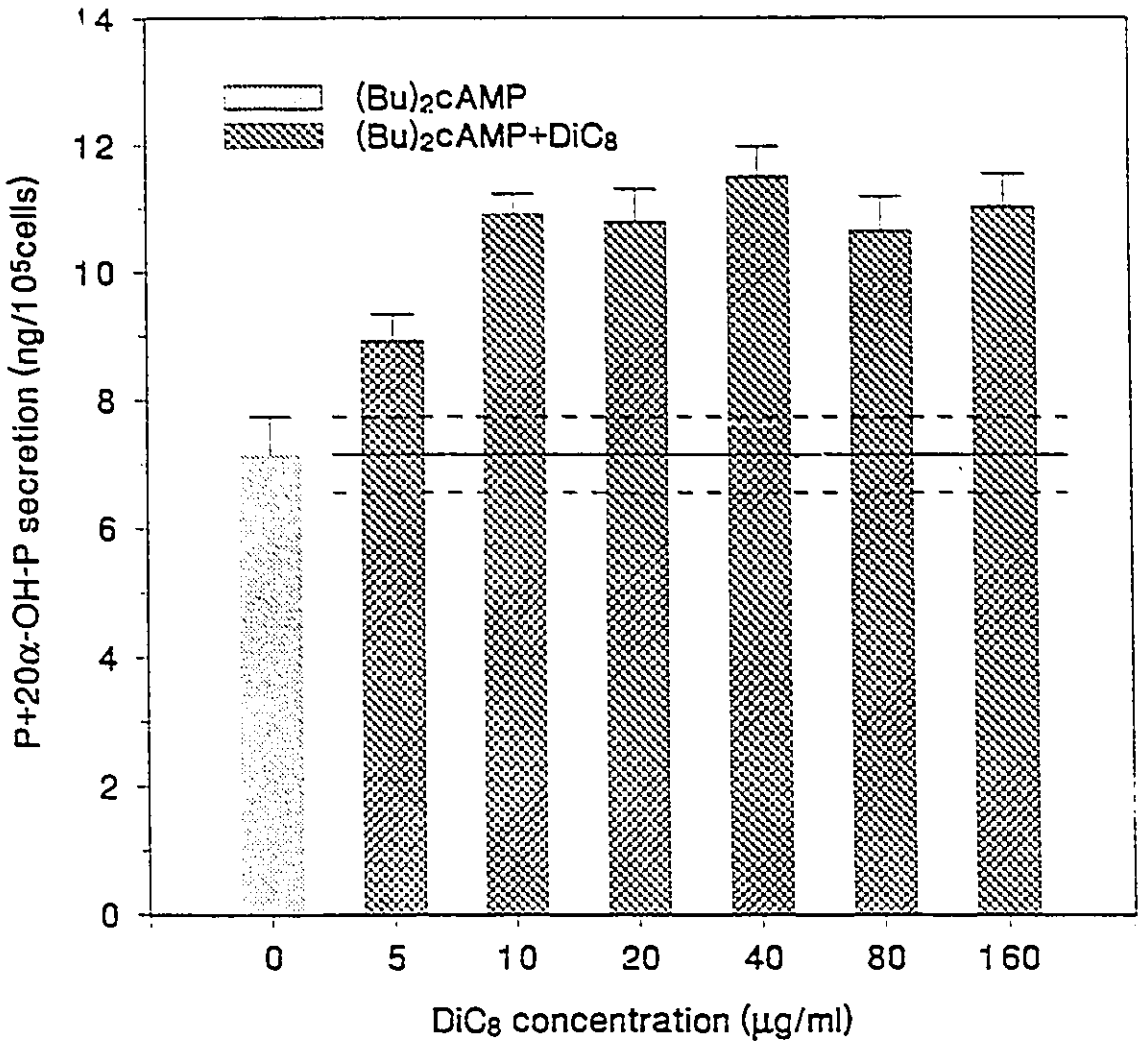

Figure 36 Concentration-dependent influence of DiC_8 on $(Bu)_2cAMP$ (1.5mM)-stimulated secretion of total progestins (P+20α-OH-P) in 24 h cultures. Values represent means ±SEM of 9 incubations from 3 experiments.

Table 13 Lack of effect of diolein on $(Bu)_2$cAMP (1.5 mM)-stimulated total progestin (P+20α-OH-P) secretion during a 24 h culture period.

Treatment Groups	P + 20α - OH-P secretion (ng/10^5cells)
Control	1.22 ± 0.11
$(Bu)_2$cAMP (1.5μM)	6.56 ± 0.58 [a]
$(Bu)_2$cAMP (1.5μM) + Diol (2.5μg/ml)	5.73 ± 0.60 [a]
$(Bu)_2$cAMP (1.5μM) + Diol (5μg/ml)	7.55 ± 1.20 [a]
$(Bu)_2$cAMP (1.5μM) + Diol (10μg/ml)	7.07 ± 0.35 [a]
$(Bu)_2$cAMP (1.5μM) + Diol (20μg/ml)	6.87 ± 0.85 [a]
$(Bu)_2$cAMP (1.5μM) + Diol (40μg/ml)	5.59 ± 0.62 [a]

Values represent means ± SEM of 6 incubations from 2 experiments.

[a] - $p <$ (compared to control)

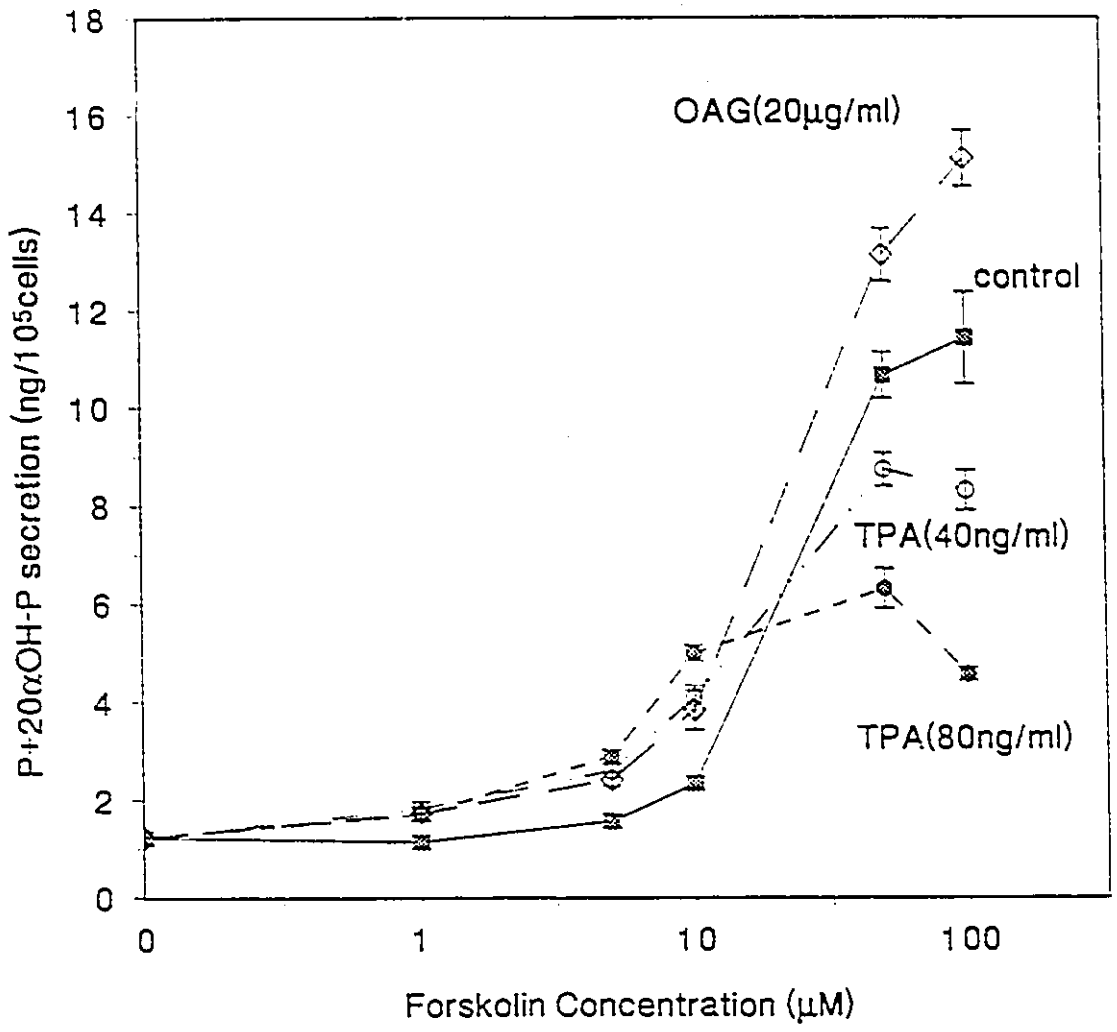

Figure 37 Influence of OAG (20 µg/ ml) and TPA (40 and 80 ng/ml) on forskolin (1-100µM)-stimulated total progestin (P+20α-OH-P) secretion in 24 h cultures. Values represent means ±SEM incubations from 2 experiments.

III. WHAT ARE THE POSSIBLE REASONS FOR THE DIFFERENTIAL EFFECTS OF DIACYLGLYCEROLS AND TPA ON GRANULOSA CELL STEROIDOGENESIS IN VITRO?

The present studies on the role of PKC in the regulation of granulosa cell steroidogenesis have demonstrated that synthetic diacylglycerols and tumor promoting phorbol ester exerted divergent effects on FSH-stimulated secretion of P_5, P and 20α-OH-P. Although the concentrations of OAG, DiC_8 and diolein present in the current studies were much higher than those of TPA, it was unclear whether the experimental conditions (e.g. duration of incubation) were adequate to ensure that a sufficient concentration of each diacylglycerol reached the site of enzyme activation prior to their metabolism. In addition, since PKC isoforms are known to be present in a variety of tissues, the possibility that the divergent effects of OAG and TPA on gonadotropin-stimulated steroidogenesis in granulosa cells were due (at least in part) to the activation of different isoenzymes of the PKC family, could not be excluded. To explore these suppositions, the steroidogenic effects of OAG and TPA, alone or in combination, were compared in the presence and absence of FSH.

A. Effect of 1 h Pretreatment of Granulosa Cells with OAG and TPA on Subsequent Basal and FSH-Stimulated Total Progestin (P + 20α - OH-P) Secretion

As shown in Fig. 38, 1 h pretreatment of granulosa cells with OAG (20 μg/ml) had no influence on basal or FSH (150 ng/ml) - stimulated steroidogenesis during 6 h or 24 h incubation. However, 1 h pretreatment with TPA (40 ng/ml) significantly increased basal but inhibited FSH-stimulated total progestin (P + 20α - OH-P) secretion in both culture periods studied, with greater inhibition (~55%) noted at 24 h.

B. Influence of the Duration of Pretreatment with OAG on Basal and FSH-Stimulated Steroidogenesis.

To determine if the duration of pretreatment of granulosa cells with OAG was limiting the effect of the PKC activator on steroidogenesis, the cells were preincubated with OAG (20 μg/ml) for 1, 6, and 12 h, and further incubated until a total culture period reached 24 h. As shown in Table 14, regardless of the duration of the preincubation period (1-12 h), OAG (when not present in culture medium continuously) failed to exert any significant effect on subsequent basal or FSH-stimulated progestin secretion

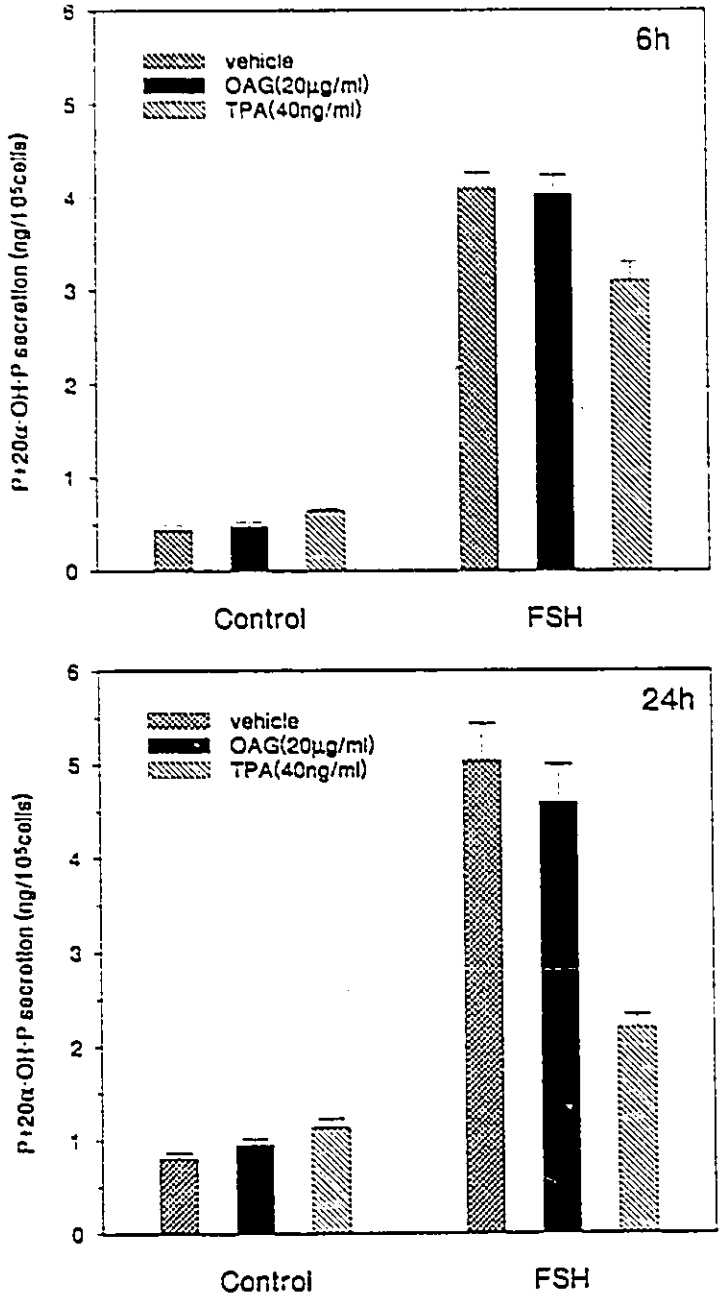

Figure 38 Influence of 1 h pretreatment with OAG (20 µg/ml) or TPA (40 ng/ml) on subsequent basal and FSH-stimulated secretion of total progestins (P+20α-OH-P) during a 6 h and 24 h incubation period. Values represent means ±SEM of 9 incubations from 3 experiments.

Table 14 Influence of 1 - 12 h preincubation with OAG (20 µg/ml) on subsequent basal and FSH (150 ng/ml)-stimulated total progestin (P+20α-OH-P) secretion during 12 - 23 h incubation period.

Preincubation (hours)	Incubation (hours)	Progestin (P + 20α - OH-P) secretion (ng/10^5 cells)			
		Control		FSH - stimulated	
		MEM pretreated	OAG (20µg/ml) pretreated	MEM pretreated	OAG (20µg/ml) pretreated
1h	23 h	1.88 ± 0.2	2.05 ± 0.2	6.05 ± 0.3	6.46 ± 0.5
6 h	18 h	1.75 ± 0.18	1.75 ± 0.18	4.52 ± 0.02	5.21 ± 0.04
12 h	12 h	0.93 ± 0.09	0.90 ± 0.06	3.06 ± 0.09	3.17 ± 0.08

Values represent means ± SEM of 6 incubations from 2 experiments.

C. Influence of OAG and TPA, Alone or in Combination, on Basal and FSH-Stimulated Total Progestin (P+20α-OH-P) Secretion.

OAG (20 μg/ml) increased significantly ($p<0.05$) total progestin (P+20α-OH-P) secretion during a 24 h- but not a 6 h- incubation period (Table 15). TPA alone, however remained ineffective during both incubation periods ($p>0.05$). In the presence of OAG (20 μg/ml) and TPA (40 ng/ml) added together, total progestin (P+20α-OH-P) secretion was increased significantly (vs. control; $p<0.05$) irrespective of the duration of incubation (Table 15)

FSH (150 ng/ml)-induced steroidogenesis during 6 h and 24 h incubations was significantly ($p<0.05$) enhanced by OAG (20 μg/ml; 26% and 40% respectively), but markedly inhibited ($p<0.05$) by TPA (40ng/ml; 63% and 58% respectively) (Fig. 39). In the presence of both PKC activators, FSH-stimulated progestin secretion was significantly ($p<0.05$) reduced during 6 h but not 24 h incubations and was considerably lower than in the presence of OAG alone in both incubation periods. The effects of OAG and TPA appeared to be additive and significantly ($p<0.05$) different from those of OAG or TPA cultured with FSH separately (Fig. 39).

D. Influence of 1 h Pretreatment of Granulosa Cells with TPA on Subsequent Total Progestin (P+20α-OH-P) Secretion in the Presence of OAG, FSH and FSH+OAG.

OAG (20 μg/ml) significantly increased basal progestin secretion during 6 h and 24 h culture periods with a greater stimulation (190%; $p<0.05$) noted at 24 h. Pretreatment of granulosa cells with TPA (40 ng/ml) significantly ($p<0.05$) enhanced basal and OAG (20 μg/ml) -stimulated total progestin secretion during 6 h but not 24 h incubation (Fig. 40).

FSH (150 ng/ml) markedly ($p<0,05$) increased progestin secretion at 6 h and 24 h (10- and 5-fold, respectively; Fig. 40 vs Fig. 41). However, the influence of FSH was not affected by the presence of OAG (20 µg/ml) (Fig. 41). Pretreatment of the cells with TPA (40 ng/ml) for 1 h significantly ($p<0,05$) inhibited FSH-stimulated progestin secretion in the presence or absence of OAG during a subsequent 24 h culture (Fig. 41). Although OAG failed to increase FSH-stimulated progestin secretion during 24 h, it significantly ($p<0,05$) diminished the inhibitory effect of TPA pretreatment.

Table 15 Influence of OAG (20 µg/ml) and TPA (40 ng/ml) alone or in combination on basal secretion of total progestins (P+20α-OH-P) during a 6 h and 24 h incubation period.

	P + 20α - OH-P secretion (ng/10^5 cells)	
Treatment Groups	6 h incubation	24h incubation
Control	0.70 ± 0.09	1.20 ± 0.10
OAG (20 µg/ml)	0.86 ± 0.06	2.35 ± 0.18[a]
TPA (40 ng/ml)	0.85 ± 0.05	1.03 ± 0.11
OAG(20µg/ml)+TPA(40 ng/ml)	1.14 ± 0.10[a.]	2.01 ± 0.19[a.b]

Values represent means ± SEM of 9 incubations from 3 experiments.

[a] - $p < 0.05$ (compared to control)
[b] - $p < 0.05$ (compared to TPA alone)

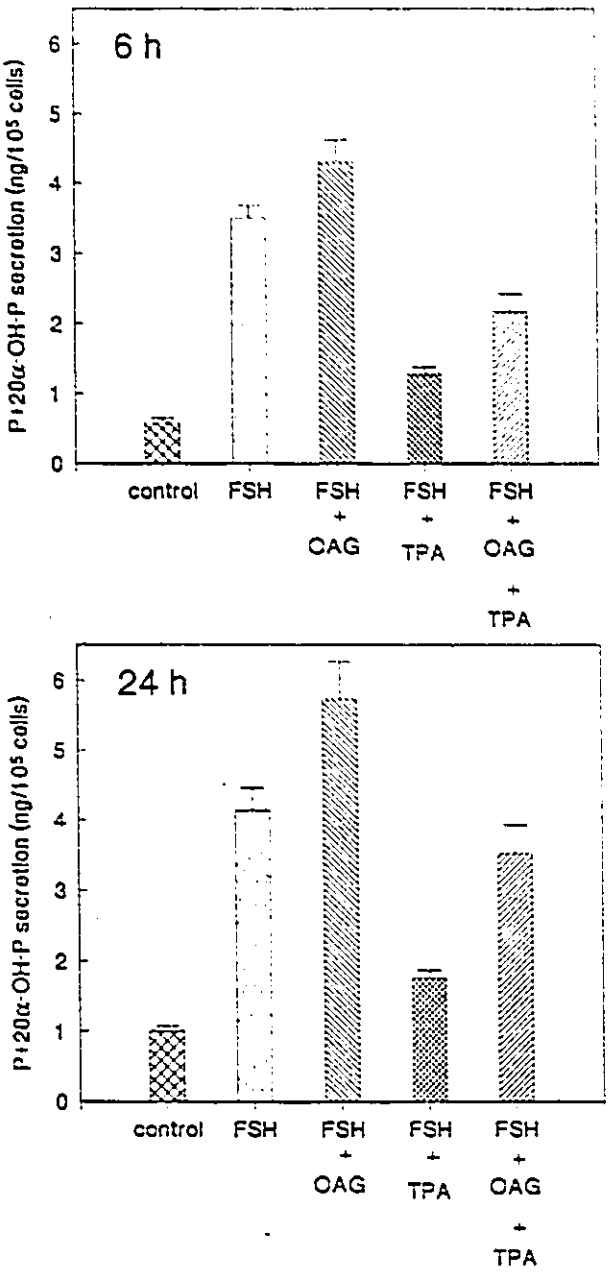

Figure 39 Influence of cotreatment of granulosa cells with OAG (20 µg/ ml) and TPA (40 ng/ml) on FSH (150 ng/ml)-stimulated secretion of total progestins (P+20α-OH-P) during a 6 h and 24 h incubation period.. Values represent means ±SEM of 6 incubations from 2 experiments.

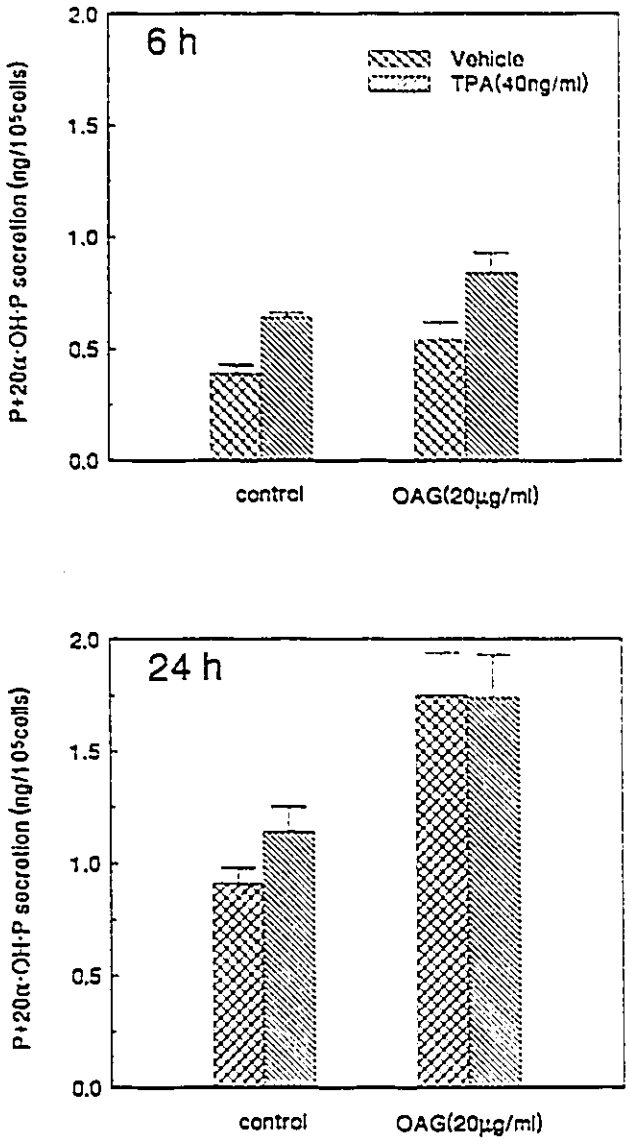

Figure 40 Influence of 1 hour preincubation with TPA (40 ng/ml) on subsequent basal and OAG (20 µg/ml) - stimulated secretion of total progestins (P+20α-OH-P) during a 6 h and 24 h incubation period. Values represent means ±SEM of 9 incubations from 3 experiments.

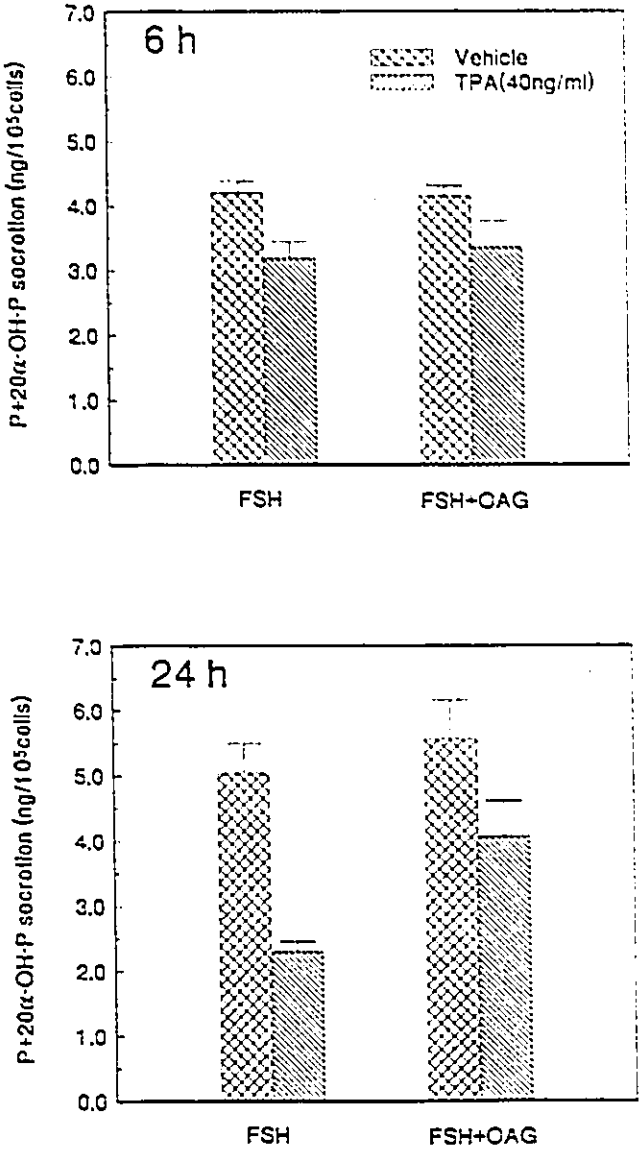

Figure 41 Influence of 1 hour preincubation with TPA (40 ng/ml) on subsequent FSH (150 ng/ml)- and FSH (150 ng/ml) plus OAG (20 µg/ ml)-stimulated progestin secretion during a 6 h and 24 h incubation. Values represent means ±SEM of 9 incubations from 3 experiments.

E. Influence of 20 h Preincubation of Granulosa Cells with TPA on OAG-, FSH-, and OAG + FSH-Stimulated Total Progestin (P+20α-OH-P) Secretion.

The influence of 20 h preincubation of granulosa cells with TPA (known to down-regulate PKC during its prolonged presence in a tissue culture) on the subsequent OAG stimulation (6 h) of total progestin (P+20α-OH-P) secretion was examined. As shown in Table 16, OAG (20 µg/ml) and TPA (40ng/ml) significantly ($p<0,05$) stimulated progestin secretion during a 6 h incubation, preceded by preincubation with MEM alone. Pretreatment with TPA (40 ng/ml) did not affect basal secretion of progestins during the subsequent 6 h but prevented any stimulation by OAG and TPA (Table 16), thus holding steroid secretion at the level of the controls.

FSH (150 ng/ml)-stimulated secretion of P+20α-OH-P was not significantly ($p>0,05$) affected by the presence of OAG (20 µg/ml) during a 6 h incubation subsequent to 20 h pretreatment with MEM only. Preincubation with TPA (40 ng/ml) significantly inhibited FSH-, (FSH + OAG)- and (FSH + TPA)-stimulated secretion of progestins (Table 16)

Table 16 Influence of 20 h preincubation with TPA (40 ng/ml) on subsequent basal, OAG (20 µg/ml)-, FSH (150 ng/ml)- and OAG plus FSH-stimulated secretion of total progestins (P+20α–OH-P) in 24 h incubation.

	P + 20α-OH-P secretion (ng/10^5 cells)	
Treatment Groups	MEM pretreatment	TPA pretreatment
Control	1.10 ± 0.10	0.83 ± 0.06
OAG(20µg/ml)	1.90 ± 0.12[a]	1.15 ± 0.14[b]
TPA(40ng/ml)	1.62 ± 0.09[a]	0.95 ± 0.15[e]
FSH(150ng/ml)	4.70 ± 0.60[a]	2.66 ± 0.34[ac]
FSH(150ng/ml) + OAG(20µg/ml)	4.95 ± 0.38[a]	2.53 ± 0.25[ad]
FSH(150ng/ml) + TPA(40ng/ml)	3.55 ± 0.25[a]	2.13 ± 0.17[f]

Values represent means ± SEM of 9 incubations from 3 experiments.

[a] - $p < 0.05$ (compared to control)
[b] - $p < 0.05$ (compared to OAG alone in MEM pretreatment group)
[c] - $p < 0.05$ (compared to FSH alone in MEM pretreatment group)
[d] - $p < 0.05$ (compared to FSH + OAG in MEM pretreatment group)
[e] - $p < 0.05$ (compared to TPA alone in MEM pretreatment group)
[f] - $p < 0.05$ (compared to FSH + TPA in MEM pretreatment group)

F. Effect of A23187 on Secretion of P_5, P and 20α-OH-P

A23187 (0.25-1 μg/ml) significantly ($p<0.05$) stimulated granulosa cell P and 20α-OH-P but not P_5 secretion during a 24 h culture period (Table 17). The effect of the calcium ionophore was concentration-dependent, with maximal increase (~2-fold) of P and 20α-OH-P secretion noted at 0.5 ng/ml.

G. Influence of OAG, TPA and A23187 on Conversion of P_5 to P and 20α-OH-P

Since Ca^{2+} is believed to play an important role in the metabolism of P_5 to P (Tsang et al., 1989), the effect of OAG and TPA on the A23187-induced conversion of exogenous P_5 to P was also examined in the presence of 0.75 mM AGP (to inhibit production of endogenous P_5). A23187 dose-dependently stimulated the conversion of exogenous P_5 to P, with a significant ($p<0.05$) increase observed at 0.25 μg/ml (Fig. 42). OAG alone (5-80 ng/ml), however, exerted a biphasic response on the metabolism of P_5 to progestins, as evident by a 3-fold stimulation at 5-10 mg/ml and a smaller to nonexistant response at higher concentrations (Figs. 20 and 43). Submaximal concentration of A23187 (0.25 mg/ml) completely attenuated the stimulatory effect of OAG on P and 20α-OH-P synthesis from exogenous P_5 (Fig. 43).

Although TPA (5-80 ng/ml) alone inhibited the conversion of exogenous P_5 to P, it slightly but significantly ($p<0.05$) increased the conversion of P to 20α-OH-P (Fig. 21). When added to granulosa cells in the presence of the calcium ionophore (0.25 mg/ml), TPA significantly attenuated the stimulatory effect of A23187 ($p<0.05$) on both P and 20α-OH-P secretion (Fig. 44)

Table 17 Effects of various concentrations of A23187 on P_5, P and 20α-OH-P secretion during a 24 h culture period.

Treatment Groups	Secretion (ng/10^5 cells)		
	P_5	P	20α - OH-P
Control	0.08 ± 0.02	0.28 ± 0.02	0.72 ± 0.08
A23187 (0.25 µg/ml)	0.09 ± 0.01	0.49 ± 0.03 [a]	1.09 ± 0.09 [a]
A23187 (0.5 µg/ml)	0.13 ± 0.02	0.67 ± 0.07 [a]	1.83 ± 0.11 [a]
A23187 (1 µg/ml)	0.06 ± 0.01	0.49 ± 0.04 [a]	1.47 ± 0.13 [a]

Values for P_5 secretion represent means ± SEM of 8 incubations from 2 experiments, and for P and 20α-OH-P secretion - 9 incubations from 3 experiments.

[a] - $p < 0.05$ (compared to control)

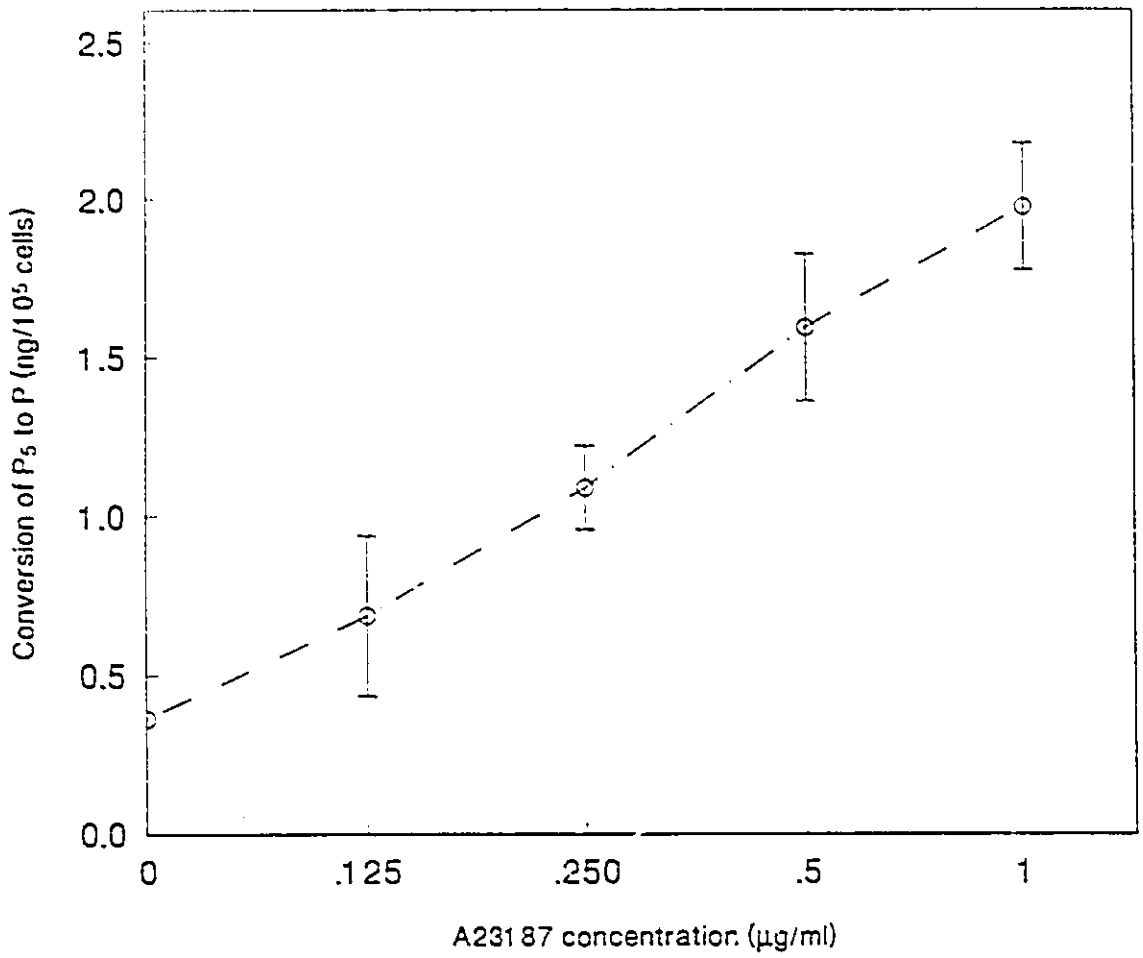

Figure 42 Dose-dependent effect of A23187 on the conversion of exogenous P_5 (0.1 µM) to P in the presence of 0.75 mM of AGP. Values represent means ±SEM of 12 incubations from 4 experiments.

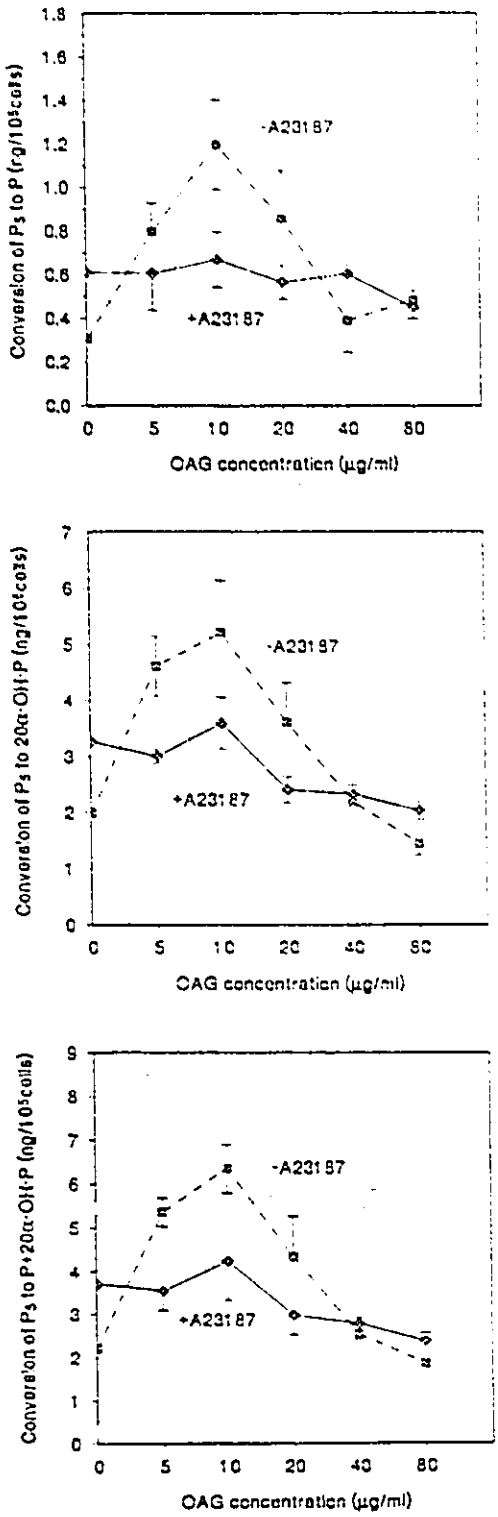

Figure 43 Effects of cotreatment of granulosa cells with OAG (0 - 80 ng/ml) and A23187 (0.25 µg/ml) on the conversion of exogenous P_5 (0.1 µM) to P and 20α-OH-P in 24 h cultures.

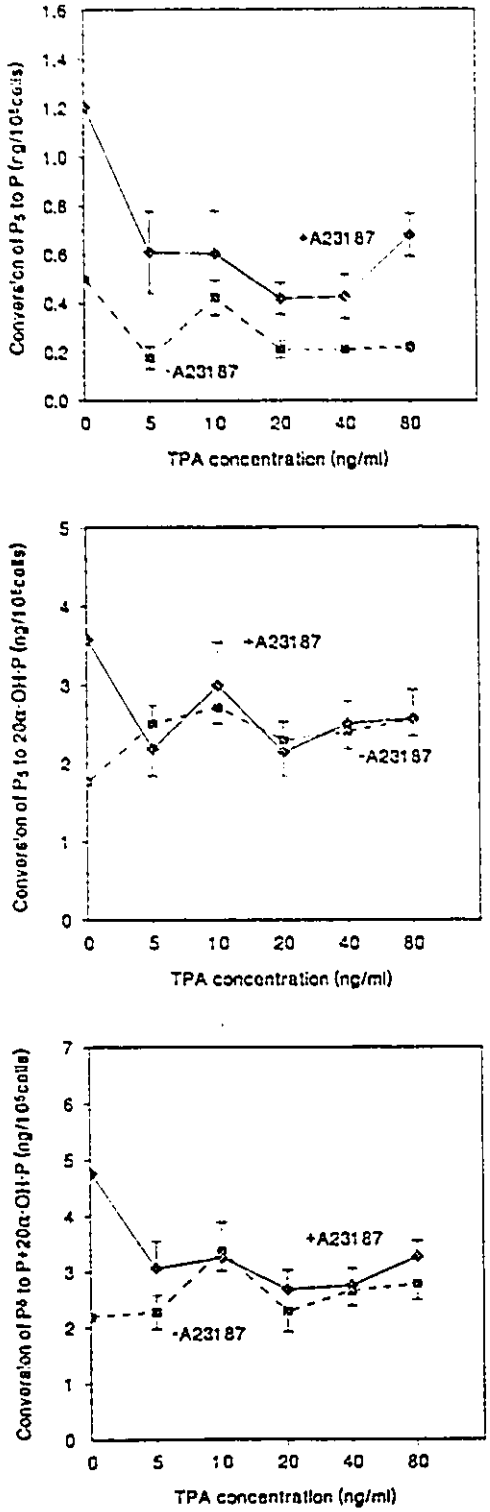

Figure 44 Effects of cotreatment of granulosa cells with TPA (0 - 80 ng/ml) and A23187 (0.25 µg/ml) on the conversion of exogenous P_5 (0.1 µM) to P and 20α-OH-P during a 24 h incubation period.

DISCUSSION

I. IS PKC INVOLVED IN THE REGULATION OF RAT GRANULOSA CELL STEROIDOGENESIS IN VITRO? IF SO, WHICH STEP(S) IN THE STEROIDOGENIC PATHWAY IS (ARE) PKC DEPENDENT?

PKC activity has been determined in rat (Clark et al., 1980, 1985; Davis and Clark, 1982; Shinohara et al., 1985) and porcine (Noland and Dimino, 1986) granulosa cells, bovine (Davis and Clark, 1983), human (Clark et al., 1983) and swine (Wheeler and Veldhuis, 1987) luteal cells and in rat ovarian theca-interstitial cells (Hofeditz et al., 1988). Low PKC activity in granulosa cells from immature, PMSG-treated rats has been reported by Clark et al. (1980) and Davis (Davis and Clark, 1982) who also noticed less activity of the enzyme in cytosol of rat granulosa cells than that in bovine or human cytosol (Davis and Clark, 1983; Clark et al., 1983).

My unsuccessful, preliminary attempts to demonstrate directly PKC activity in the rat granulosa cells prompted the present investigation of a possible involvement of the enzyme in the regulation of ovarian steroidogenesis with PKC activators and inhibitors. Synthetic, permeable diacylglycerols (OAG and DiC_8) as well as TPA significantly increased secretion of P_5, P and 20α-OH-P by granulosa cells in vitro. The effect of PKC activators was concentration-dependent and differed with respect to the duration of cultures. While OAG stimulated steroid production as early as 3-6 h of incubation, the maximal increase was observed at 12-24 h. TPA stimulated P_5 and P secretion maximally at 3-6 h, but became inhibitory with time (~50% inhibition at 24 h). Only 20α-OH-P secretion was significantly increased by both PKC activators throughout all culture peric studied. The present demonstration of the acute stimulatory action of TPA and OAG on steroidogenesis in rat granulosa cells are in agreement with earlier reports. TPA has been

shown to increase basal P_5 (Welsh et al., 1984; Kawai and Clark, 1986), P (Kawai and Clark, 1985, 1986; Shinohara et al., 1986; Wang and Leung, 1987) and 20α-OH-P (Welsh et al., 1984; Kawai and Clark, 1986) secretion by rat granulosa cells incubated up to 6 h. Relatively less is known regarding the steroidogenic effects of diacylglycerols, nevertheless available data indicate that these PKC activators and TPA have similar influence on basal progestin production during short-term incubations (Shinohara et al., 1986; Veldhuis and Demers, 1986; Wang and Leung, 1987; Tilly and Johnson, 1988). OAG (Shinohara et al., 1986) and DiC_8 (Shinohara et al., 1986; Wang and Leung, 1987) significantly stimulated P secretion by rat granulosa cells and bovine luteal cells (Brunswig et al., 1986) during short term cultures but inhibited secretion of this steroid by intact and saponine-permeabilized porcine granulosa cells (Sadighian et al., 1989).

Information concerning the delayed (24 h or more) effect of the phorbol ester and particularly that of OAG on ovarian steroidogenesis is incomplete. TPA significantly decreased basal P (Veldhuis and Demers, 1986; Hylka et al., 1989) and 20α-OH-P secretion by porcine granulosa cells incubated for 48 h, although very low concentrations of phorbol ester (< 1 ng/ml) were stimulatory (Hylka et al. 1989). The present observation that rat granulosa cell P secretion decreased during long term cultures (24 h) in the presence of comparable concentrations of TPA is consistent with the findings of Veldhuis and Demers (1986) and Hylka et al. (1989). However, while phorbol ester significantly increased 20α-OH-P secretion with no change in total progestin (P+20α-OH-P) accumulation in the rat, an inhibition of the metabolism of P in swine granulosa cells in the presence of TPA has been reported (Veldhuis and Demers 1986). Results from the present studies also differ from the observations of Sadighian et al. (1989) which demonstrated an inhibitory action of OAG in swine granulosa cells.

It is possible that differences between my results and those of Veldhuis and Demers (1986) and Sadighian et al. (1989) could be related to species-specific responses. However, differences in incubation conditions (e.g. duration of culture) and in the stage of follicular

cell differentiation cannot be excluded as possible contributing factors, since TPA and OAG are known to exert different actions on small and large granulosa and luteal cells (Shinohara et al., 1986; Brunswig et al., 1986; Hansel et al., 1987; Benhaim et al., 1990)

The present study with PKC inhibitors also suggests an involvement of the enzyme in rat granulosa cell steroidogenesis. Two specific inhibitors, H_7 and ESP, dose-dependently inhibited both OAG- and TPA-stimulated progestin secretion. These findings are in keeping with the observations of Wang and Leung (1987) and Sancho-Tello (1991), which demonstrated a reduction in TPA-stimulated steroid secretion by granulosa cells in the presence of H_7 and with the reports of Sender Baum and Ahren (1988) showing an inhibition of P accumulation in rat luteal cell suspensions by sphingosine.

PKC activators appear to influence each step of the granulosa cell steroidogenic pathway. In the present studies, OAG and TPA significantly affected the accumulation of P_5 from endogenous cholesterol (in the presence of cyanoketone to block P_5 metabolism) and the conversion of exogenous P_5 to P and 20α-OH-P (in the presence of AGP to inhibit the endogenous P_5 synthesis) as well as the conversion of exogenous P to 20α-OH-P (in the presence of cyanoketone to prevent endogenous P formation). The significant influence of OAG and TPA on these steps suggests an involvement of PKC in the regulation of the rat granulosa cell steroidogenic enzymes: $P_{-450scc}$ (irreversibly metabolizing cholesterol to P_5), 3β-HSD (irreversibly metabolizing P_5 to P) and 20α-HSD (reversibly converting P to 20α-OH-P). The biphasic action of OAG on P_5 and total progestin (P+20α-OH-P) secretion, as evident by a relatively narrow stimulatory concentration range and a declining stimulation at increased concentrations, indicates a high sensitivity of these enzymatic reactions to small changes in the cellular level of the phospholipid. Although OAG appeared to be capable of activating all three steroidogenic enzymes, its effects on $P_{-450scc}$ and 3β-HSD were particularly evident. OAG increased the accumulation of P_5 by 3-fold, the conversion of exogenous P_5 to P by about 5-fold, and the conversion of exogenous P to 20α-OH-P by less than 2-fold. In addition, the

20α-OH-P P secretion ratio was dose-dependently decreased by OAG, suggesting a greater stimulation of 3β–HSD than of 20α-HSD.

The effect of TPA on P_5 and P secretion differed in the present study from that of OAG with respect to the magnitude and duration of incubation. TPA increased P_5 and P secretion (by about 40%) at 3-6 h of incubation but inhibited the accumulation of P_5 and the conversion of exogenous P_5 to P during 24 h cultures. It is not clear whether the inhibition of P_5 accumulation by TPA was due to the reduction of $P_{-450scc}$ activity and or an effect of the phorbol ester on step(s) preceding the side chain cleavage. In addition it remains to be determined whether the reduction in P secretion at 24 h was a consequence of decreased activity of 3β-HSD as compared to that in early hours of incubation or was due to increased metabolism to 20α-OH-P in the presence of TPA. 20α-OH-P secretion and conversion of P to 20α-OH-P (in the presence of cyanoketone) were augmented by TPA during short and long culture periods. 20α-OH-P/P secretion ratio was also increased by TPA, suggesting a higher activity of 20α-HSD.

It has been reported that PKC phosphorylates sterol carrier protein (Steinschneider et al., 1989) known to mediate the transfer of cholesterol from lipid droplets to mitochondria and from the outer to the inner mitochondrial membrane, where the cholesterol side chain cleavage enzyme, cytochrome $P_{-450scc}$ is located (Vahouny et al., 1983, 1987). In rat (Trzeciak et al., 1987) and human (Tremblay et al., 1991) granulosa cells, TPA has been shown to stimulate the synthesis of the side chain cleavage enzyme complex (approximately 3-fold) during long (24 h-48 h) term cultures. Phorbol ester has also been found to either have no effect or to stimulate 3β-HSD (Welsh et al., 1984; Kawai and Clark, 1986; Chedrese et al., 1990b; Tremblay et al., 1991) and 20α-HSD (Welsh et al., 1984; Kawai and Clark, 1986) activities. The discrepancy between the present study and those of Trzeciak et al. (1987) and Tremblay (1991) regarding the effects of TPA on P_5 secretion and synthesis of SCC enzyme complex might be due to the differences in the experimental conditions (eg. pretreatment of rats, TPA concentration).

In addition, while these authors assessed the synthesis of SCC and mRNA levels I measured the production of the hormone catalyzed by the enzymes. The latter depended on a variety of factors, including substrate availability and product inhibition (cyanoketone used in the present study prevented metabolism of P_5 to P).

II. IS PKC INVOLVED IN THE STIMULATION OF GRANULOSA CELL STEROIDOGENESIS BY FSH? DOES IT PARTICIPATE IN THE MODULATION OF FSH-INDUCED STEROID HORMONE PRODUCTION?

Although FSH is believed to exert its physiological response through the adenylyl cyclase signal transducing system, an involvement of the PKC pathway in the stimulation of steroidogenesis by this gonadotropin cannot be excluded. Activation of the PKC pathway exerts marked modulatory effects on gonadotropin-stimulated steroidogenesis in vitro. Tumor promoting phorbol ester has been shown to inhibit FSH-, LH- or hCG-induced steroid production by rat (Welsh et al., 1984; Kawai and Clark, 1985; 1896; Trzeciak et al., 1986), porcine (Veldhuis and Demers, 1986; Hylka et al., 1989) and chicken (Asem and Tsang, 1988; Tilly and Johnson, 1988) granulosa cells, bovine (Brunswig et al., 1986) and rat (Sender Baum and Rosberg, 1987) luteal cells as well as rat (Hofeditz et al., 1988) and chicken (Tilly and Johnson, 1989) theca-interstitial cells. In addition it has also been found to reduce FSH-dependent aromatization of androgens to estrogens in rat Sertoli cells (Monaco and Conti, 1987) and testosterone production by mouse Leydig cells (Mukhopadhyay et al., 1984).

In the present study FSH markedly stimulated the secretion of P_5, P and 20α-OH-P. The addition of TPA to the cell incubations significantly decreased FSH-induced secretion of all three steroids in a time- and concentration-dependent manner. These results are in agreement with the earlier reports that TPA attenuates LH- and FSH-

stimulated P_5, P and 20α-OH-P secretion by granulosa cells in vitro (Welsh et al., 1984; Veldhuis and Demers, 1986; Kawai and Clark, 1986; Trzeciak et al., 1987) and that the PKC activator suppresses the gonadotropin stimulation of steroidogenic enzymes (Welsh et al., 1984; Kawai and Clark, 1986; Trzeciak et al., 1987; Chedrese et al., 1990b). TPA is known to decrease FSH-induced synthesis of $P_{-450scc}$ and to reduce mRNA levels encoding the precursor form of these proteins (Trzeciak et al., 1987). TPA has also been shown to inhibit 3β-HSD activity (Welsh et al., 1984; Kawai and Clark, 1986) and to reduce 3β-HSD mRNA levels (Chedrese et al., 1990b) induced by gonadotropins. In contrast to other steroidogenic enzymes in granulosa cells, 20α-HSD did not appear to be regulated by FSH (in vitro), but was significantly stimulated by phorbol ester irrespective of the presence of gonadotropin (Welsh et al., 1984; Kawai and Clark, 1986).

Results from my studies have shown that whereas FSH failed to significantly stimulate the conversion of exogenous P to 20α-OH-P, TPA increased it in a concentration-dependent manner. Similarly, although the 20α-OH-P/P secretion ratio remained constant in the presence of this gonadotropin, it was increased by the phorbol ester. The decrease in FSH-induced 20α-OH-P secretion by the phorbol ester could have been secondary to its inhibition of P_5 and P synthesis. These results are in keeping with the finding of Welsh et al. (1984), who demonstrated a marked reduction in rat granulosa cell 20α-OH-P production by TPA independent of 20α-HSD activity.

The inhibitory effect of TPA on FSH-stimulated P_5 and total progestin (P+20α-OH-P) secretion could not be due to cytotoxicity since basal P_5, P and 20α-OH-P secretion were markedly stimulated by the phorbol ester in the same cultures. TPA stimulated the conversion of exogenous P to 20α-OH-P. Moreover, TPA treatment did not change cell number or cell viability. The inhibitory action of phorbol ester did not appear to be due to a non-specific feature of its diterpene structure, since 4α-phorbol 12,13-didecanoate and phorbol-13-monoacetate, two phorbol esters with no tumor promoting activity, failed to significantly influence FSH-induced secretion of progestins.

Numerous studies have shown the interaction of the PKC and cAMP pathways in steroidogenic cells and tissues. Although there are some inconsistencies, the majority of reports have demonstrated that TPA has no effect on basal cAMP production (Shinohara et al., 1986; Brunswig et al., 1986; Veldhuis and Demers, 1986; Wheeler and Veldhuis, 1989) and inhibits gonadotropin-induced cAMP accumulation (Mukhopadhyay and Schumacher, 1985; Asem and Tsang, 1988; Tilly and Johnson, 1988; Wheeler and Veldhuis, 1988; Hylka et al., 1989; Davis et al., 1989). However, the activation of PKC by TPA in steroidogenic cells in vitro has also been associated with variable responses in basal and gonadotropin-induced cAMP formation (Veldhuis and Demers, 1986; Shinohara et al., 1986; Jalkanen et al., 1987; Sender Baum and Rosberg, 1987; Wheeler and Veldhuis, 1989). According to Trzeciak et al. (1987) and Chedrese et al. (1990b), the decrease in the gonadotropin-regulated progestin secretion by TPA results from the suppression of the increases in $P_{-450sec}$ and 3β-HSD enzymes content, due partially to the inhibition of gonadotropin-induced increases in cAMP levels.

Based on the findings by Shinohara et al. (1985), Trzeciak et al. (1987), Wheeler and Veldhuis (1988), Chedrese et al. (1990b) and the present observations, it appears that the effect of TPA on gonadotropin-induced steroid secretion is at the level of the synthesis and action of cAMP. There are many possible sites of interaction between PKC and adenylyl cyclase pathway. According to Wheeler and Veldhuis (1988) there is an early and a delayed increase in FSH-stimulated cAMP formation in swine granulosa cells and TPA inhibits both phases of cAMP accumulation. Moreover, two phases of FSH-induced cAMP formation have also been reported in the rat granulosa cells: the first phase lasted up to 6 h after the exposure to FSH, while the second began after 24 h of culture (Knecht et al., 1981). In granulosa cells from diethylstilbestrol-treated immature rats however, TPA prevented only the second FSH-mediated rise in cAMP accumulation (Shinohara et al., 1985). In the present studies TPA decreased FSH-induced P_5, P and 20α-OH-P secretion, with the greatest inhibition noted during the first 6 h of incubation. Twenty

hour pretreatment of granulosa cells with the tumor promoting phorbol ester reduced the subsequent induction of progestin secretion by the gonadotropin. While the basis for the temporal pattern of FSH-stimulation and its modulation by TPA remains to be determined, the possibility of an action of TPA on PKC activity (down-regulation), FSH receptor, SCP_2 and steroidogenic enzymes must be considered.

Changes in granulosa cell cAMP levels following PKC activation may be a consequence of the enzyme effect on hormone-receptor interaction (Mukhopadhyay et al., 1984; Kasson et al., 1985; Wheeler and Veldhuis, 1988) or on the G-proteins regulatory function. Experiments with bovine luteal cells have shown that hormone-responsive phospholipase C is coupled to a G-protein (Davis et al., 1988). Activation of PKC may alter the binding of GTP or Mg^{2+} to the G-protein and may affect association or dissociation of its α, β and γ subunits. In addition, PKC may modulate the interaction of the G-protein with the catalytic subunits and/or the hormone-receptor complexes (Wheeler and Veldhuis, 1988). Cholera toxin and pertussis toxin have been used extensively to probe the involvement of G_S (stimulatory) and G_I (inhibitory) proteins, respectively, in hormone signalling. In steroidogenic cells, cholera toxin significantly stimulated the secretion of steroids (Shinohara et., al, 1986; Brunswig et al., 1986; Kawai and Clark, 1986; Suh and Amsterdam, 1990; Monaco and Conti, 1987) and cAMP accumulation (Monaco and Conti, 1987; Suh and Amsterdam, 1990). Its action on cAMP however, was variable in the presence of TPA (Kawai and Clark, 1986; Shinohara et al., 1986; Veldhuis and Demers, 1986; Monaco and Conti, 1987; Wheeler and Veldhuis, 1988, 1989; Suh and Amsterdam, 1990). The results presented by Wheeler and Veldhuis (1989) in swine granulosa cells indicate that the action of PKC does not involve G_I protein, since pertussis toxin synergizes with maximal stimulatory concentrations of LH, forskolin and cholera toxin to increase cAMP production.

Although the catalytic subunit of adenylyl cyclase has also been suggested as a possible site of regulation by PKC, the effects of TPA on ovarian steroidogenesis induced

by forskolin, a stimulator of the catalytic subunit, have been variable (Veldhuis and Demers, 1986, Brunswig et al., 1986; Asem and Tsang, 1988; Wheeler and Veldhuis, 1988, Hylka et al., 1989; Suh and Amsterdam, 1990). In addition, forskolin-induced cAMP accumulation in cultured granulosa cells has been reported to be either increased (Shinohara et al., 1986; Suh and Amsterdam, 1990), decreased (Wheeler and Veldhuis, 1988; Hylka et al., 1989) or unaffected (Veldhuis and Demers, 1986) by TPA. In the present investigations, phorbol ester had a biphasic effect on forskolin-induced secretion of progestins. While TPA potentiated the action of submaximal stimulatory concentrations (0-10 μM) of forskolin on P and 20α-OH-P secretion, it was inhibitory in the presence of higher concentrations of this agent (50-100 μM). Interestingly, as observed in the present studies, a stimulatory action of phorbol ester on forskolin-induced steroidogenesis (Brunswig et al., 1986) and cAMP formation (Shinohara et al., 1986) has been reported in granulosa and luteal cells at low concentrations (<10 μM) of forskolin. Moreover, the present observations are consistent with earlier reports that TPA suppresses the increases in steroid production and cAMP accumulation induced by maximal stimulatory concentrations of the adenylyl cyclase activator (50-100 μM) (Asem and Tsang, 1988; Wheeler and Veldhuis, 1988; Hylka et al., 1989). The dependance of the effects of TPA on the magnitude of adenylyl cyclase stimulation by forskolin are in keeping with the notion that PKC modulates other signalling pathways (Berridge 1986,1987).

Studies by Kawai and Clark (1895), Shinohara et al. (1986), Brunswig et al. (1986), Suh and Amsterdam (1990) as well as the present observations suggest that PKC acts at the site of and distal to cAMP formation. In addition, investigations using TPA as a probe for PKC involvement in the regulation of steroidogenesis have provided evidence dissociating the steroid secretory activity of the cell from the cAMP response, thus indicating possibly the presence of steroidogenic mechanisms independent of cAMP generation. In rat (Kawai and Clark, 1985; Trzeciak et al., 1987) and swine (Veldhuis and

Demers, 1986) granulosa cells, phorbol ester significantly influenced P secretion without affecting cAMP formation (Kawai and Clark, 1985; Trzeciak et al., 1987; Veldhuis and Demers, 1986). Experiments with highly steroidogenic rat granulosa cells cotransfected with SV_{40} DNA and Ha-ras oncogene have shown that while forskolin- and cholera toxin-induced cAMP production was increased by TPA, P and 20α-OH-P secretion were inhibited. Veldhuis and Demers (1986) also reported no significant change in FSH-, forskolin- or cholera toxin-induced cAMP accumulation when progestin secretion was markedly inhibited by TPA during prolonged (48 h) cultures.

Exogenous cAMP such as 8-bromo cAMP and $(Bu)_2$cAMP have been used extensively to test the ability of phorbol ester to modulate steroid production at steps following cAMP formation. In the present studies, $(Bu)_2$cAMP markedly increased P_5, P and 20α-OH-P secretion. However, the addition of TPA to granulosa cells inhibited $(Bu)_2$cAMP-induced steroidogenesis in a time- and concentration-dependent manner. These observations are in agreement with the inhibitory action of TPA on cAMP-stimulated P secretion noted in rat (Kawai and Clark; Trzeciak et al.), swine (Veldhuis and Demers, 1987; Hylka et al., 1989) and chicken (Asem and Tsang, 1989; Tilly and Johnson, 1988) granulosa cells, bovine luteal cells (Brunswig et al., 1986) and in highly steroidogenic granulosa cell lines (Suh and Amsterdam, 1990). The mechanism(s) by which phorbol ester influences cyclic nucleotide-regulated steroidogenesis is (are) not clear. One possible site of TPA action may involve cAMP-regulated genes. It has been demonstrated that transcription factors AP-1 (Lee et al., 1987; Angel et al., 1987) and AP-2 (Imagawa et al., 1987) mediate transcriptional induction by phorbol ester and that rapidly induced genes of the *jun* family are modulated in an antagonistic manner by TPA and cAMP (Chin et al., 1988; Curran and Franza, 1988). Since two members of the *Jun* gene family have recently been identified in the ovary (Hirari et al., 1989), it is tempting to speculate that TPA might be blocking some of the genes turned on by cAMP. Another

possible mechanism of TPA action may be related to the post-translational phosphorylation of key proteins that offset the action of cAMP (Hirari et al., 1989).

Although the inhibitory effect of TPA on FSH-induced P_5, P and 20α-OH-P secretion appears to be, at least partially, due to the modulatory action of PKC on the gonadotropin-stimulated cAMP pathway, a role of PKC as one of the mediators of FSH action should also be considered. In the present study prolonged pretreatment of granulosa cells with TPA (known to down-regulate PKC) significantly reduced subsequent FSH induction of progestin secretion by the gonadotropin. Also PKC inhibitors (H_7 and ESP) similarly decreased FSH-stimulated steroidogenesis, implying a possible involvement of PKC in the FSH action on granulosa cell progestin secretion. The PKC inhibitor ESP, at the concentrations not affecting cell viability, did not seem to influence cAMP production or action, since it did not have any significant effect on forskolin- or $(Bu)_2$cAMP-stimulated steroidogenesis.

Of particular importance in the present studies is the demonstration of the divergent effects of OAG and TPA on FSH-stimulated steroidogenesis in granulosa cells in vitro. In contrast to the inhibitory action of TPA evident within 1 h of pretreatment, OAG potentiated FSH-induced secretion of P_5, P and 20α-OH-P, albeit only if the diacylglycerol was continually present in the culture medium. The extent of the enhancement of progestin secretion induced by diacylglycerol depended on the magnitude of stimulation by the gonadotropin: the smaller the increase elicited by FSH, the greater was the enhancement of steroid secretion by OAG. There seemed to be a maximal level of stimulation by the gonadotropin (~5 ng/10^5 cells), above which no further enhancement could be evoked with diacylglycerol, regardless of its concentration. The effect of OAG was not specific for this particular diacylglycerol, since DiC_8 not only caused a significant but also a greater enhancement of progestin secretion.

The mechanism(s) of action of exogenous diacylglycerols on steroid production by granulosa cells have not been studied thoroughly. Since it is commonly assumed that

OAG and TPA evoke similar physiologic responses, the latter has been used primarily as a substitute for diacylglycerol. Gonadotropin-stimulated P secretion has been shown to be increased in rat (Shinohara et al., 1986) but inhibited in swine (Veldhuis and Demers, 1986) granulosa cells by diacylglycerols during a 2 h incubation. In hen granulosa cells OAG decreased LH-stimulated P secretion (during 4 h culture). The inhibition however, was much smaller than that in the presence of TPA (~30% vs 70%). In my studies, OAG enhanced FSH-induced P_5 secretion within 1 h of incubation. Also, progestin secretion was significantly enhanced by OAG throughout all culture periods studied, although, a greater stimulation was usually observed during 24 h cultures.

Like TPA, diacylglycerols seem to act both at the site of and distal to cAMP formation, albeit in an opposite manner. OAG and DiC_8 have been shown to modulate cAMP-dependent and independent steroidogenesis. In rat granulosa cells, OAG significantly stimulated hCG- and forskolin-induced cAMP production within 15 min (Shinohara et al., 1986). The effect of diacylglycerol was short-lived and was undetectable by 2 h. That OAG functions as a genuine PKC activator is consistent with the report demonstrating the stimulatory effect of exogenous phospholipase C on granulosa cell progestin and cAMP production, which is similar to that of OAG (Shinohara et al., 1986). In the present studies, OAG dose-dependently potentiated the stimulatory action of forskolin during 24 h cultures. It is possible that the increase in granulosa cell cAMP concentration as reported by Shinohara et al. (1986) could have triggered events leading to this latent effect. Indeed, $(Bu)_2$cAMP-induced secretion of progestins was also stimulated by OAG and DiC_8, albeit only during 24 h cultures. This delayed effect of diacylglycerols could have been due to their action at the transcriptional/translational level, e.g., modulation of gene expression in the synthesis of enzymatic proteins.

III. WHAT ARE THE POSSIBLE REASONS FOR THE DIFFERENTIAL EFFECTS OF DIACYLGLYCEROLS AND TPA ON GRANULOSA CELL STEROIDOGENESIS IN VITRO?

The present study represents the first report on the differential effects of OAG and TPA on ovarian steroidogenesis. However, it is of interest to note that while OAG has been shown to enhance sphingomyelin hydrolysis and phosphatidylcholine metabolism in pituitary cells, TPA was inhibitory in this regard (Kolesnik, 1987, Kolesnik and Paley, 1987). The production of phosphatidylethanol in lymphocytes was stimulated by TPA through the activation of phospholipase D (PLD), however OAG and DiC_8 had no effect on the activity of the lymphocyte enzyme (Cao et al., 1990). Several groups of researchers have reported an inability of 1,2-diacylglycerols to mimic the action of TPA on the differentiation of the human promyelocytic cell line, H-60 (Kreutter et al., 1985; Yamamoto et al., 1985; Morin et al., 1987). In addition, Bijleveld et al. (1988) observed dissimilar effects of OAG and TPA on fatty acid synthesis in isolated rat liver cells.

There are a number of reasons for the different effects of permeable diacylglycerols and TPA on basal and FSH-regulated P_5 and P secretion by granulosa cells. It is possible that the divergent influence of these PKC activators, particularly on basal steroid secretion, are in part due to differential regulation of the enzyme per se. OAG and TPA have been shown to have dissimilar influence on PKC activity (Shinohara et al. 1985; Cochet et al. 1986 a, b; Kolesnik and Clegg, 1988), probably due to differences in their half-life and bio-availability. It is of interest to note in the present study that basal steroid secretion was increased when granulosa cells were pretreated with TPA for 1 h but was decreased when pretreatment was prolonged. FSH-induced steroidogenesis continued to be inhibited after the removal of phorbol ester from the culture medium. OAG stimulated steroid secretion only during its continual presence but, in contrast to TPA, was ineffective even when the cells were preincubated with this phospholipid for up to 12 h.

While OAG is produced in the cell transiently and both endogenous and exogenous diacylglycerols (Nishizuka et al., 1984 a,b) are rapidly converted to phosphatidic acid, TPA is metabolized slowly and remains in the cell for a prolonged period of time (Nishizuka et al., 1984 a, b; Takai et al., 1984; Berridge, 1987). During OAG and DiC_8 stimulation, PKC is believed to translocate from the cytosol to the cell membrane, but to return thereafter to the cytosol to replenish the cytosolic kinase pool (Shinohara et al., 1985; Kolesnik and Clegg, 1988). In contrast, TPA stimulates the intracellular redistribution of PKC by inserting the enzyme into a hydrocarbon region of the membrane and enhancing the formation of an irreversible complex (Bazzi and Nelsestuen, 1988), without recycling the enzyme to the cytosol (Kolesnik and Clegg, 1988). Cochet et al. (1985, 1986) have demonstrated not only the subcellular redistribution of PKC following TPA treatment, but also striking alterations in the catalytic properties of the enzyme induced by phorbol esters. Whereas total PKC activity (cytosolic and membrane-associated) was conserved following OAG stimulation, prolonged incubation of cells with TPA down-regulated the enzyme and caused a significant loss of its activity (Cochet et al., 1986 a, b; Fournier and Murray, 1987; Bazzi and Nelsestuen, 1988; Kolesnik and Clegg, 1988). It is possible that inhibition of P_5 and P secretion observed in the present study during prolonged incubation with TPA may be due in part to an alteration of kinase activity. Since exogenous diacylglycerols are metabolized like endogenous ones (Nishizuka et al., 1984 a, b) it is possible that they may function in a manner similar to that of the lipids released in response to a physiological stimulus.

Although OAG and TPA have common effects on basal steroid production by granulosa cells during short incubation, they differ in their influence on gonadotropin-stimulated steroidogenesis. A possible reason for their differential actions might be the selective activation and down-regulation of various isoforms of PKC which have been localized in a variety of intracellular compartments. In addition, the isoforms of PKC might have different activator affinity and substrate specificity (Kiley et al. 1990). It has

been shown that mammalian cells contain at least seven immunologically distinct isoenzymes of phospholipase C (Rhee et al., 1989; Crooke and Bennet, 1989). These isozymes are dissimilar not only in molecular size and amino acid sequences but are expressed differently between and within tissues and during development. The suggestion that the diversity of responses to a variety of external stimuli may be attributed to differential regulation of these isoenzymes is supported by the observation that PLC isoforms are differentially phosphorylated in response to various extracellular stimuli (Ryu et al. 1990). In addition, a number of isoenzymes of PKC have been discovered recently (Coussens et al., 1986; Hidaka et al., 1988; Nishizuka, 1988; Yoshida et al., 1988; Ohno et al., 1988; Pelosin, et al., 1990), including 3 isoforms in swine granulosa cells (Wheeler and Veldhuis, 1989). These isoenzymes seem to be distinctively distributed in different subcellular locales and their expression appears to be differentially controlled during development (Ramsdell et al., 1986; Brooks et al, 1987; Yoshida et al., 1988).

Although remained to be verified, the probability that the control of granulosa cell steroidogenesis is via differential phosphorylation of key proteins by PKC isoenzymes is of interest. In this context, Kiss and Luo (1986) and Cochet et al. (1986a) reported that OAG and TPA have markedly different effects on liver plasma membrane phosphorylation in vitro. In addition, various PKC activators including OAG, TPA and bryostatins have been shown to elicit different responses on prolactin synthesis and release in GH_4 pituitary cells (Ramsdell et al., 1986) and to have distinct phosphorylation patterns in GH_3 cells (Brooks et al., 1987). In the present study, the additive effect of OAG and TPA cotreatment or short pretreatment of cells with a maximal stimulatory concentration of TPA on steroid secretion induced by either PKC activator is consistent with a possible involvement of different PKC isoforms in the regulation of steroidogenesis. However, pretreatment with TPA for 20 h, a condition known to down-regulate the enzyme, prevented the subsequent stimulation by OAG of both basal and FSH-induced steroidogenesis. It is possible that in short incubations TPA binds to PKC isoform(s)

which is (are) more readily accessible and/or with affinity to both TPA and OAG. TPA bound to those isoform(s) with higher a affinity for diacylglycerol may readily be displaced by OAG as the latter enters the cells. However, it is conceivable that during longer cultures with TPA alone, the phorbol ester is accessible to all intracellular locations and binds to PKC isoforms of all affinities, eventually down-regulating the enzyme and rendering the cells unresponsive to OAG as was observed after 20 h pretreatment with the phorbol ester. It has been demonstrated in GH_4C_1 rat pituitary cells that PKC isoforms have different affinities for two of their activators: phorbol dibutyrate (PDBu) and mezerin (Kiley et al., 1990). The population of these PKC isoforms seemed to be homogenous to PDBu, but not to mezerin. Prolonged treatment of GH cells with PDBu inhibited two of three isoforms of PKC and 50% of the third, albeit treatment of the same cells with TRH (one of the few physiological PKC activators known to down-regulate PKC) selectively inhibited only one of the PKC isoforms and stimulated secretion of prolactin (Kiley et al., 1990). The presence of PKC in different subcellular locales may also be an important factor contributing to the divergent effects of various PKC activators on granulosa cell steroidogenesis. Isoforms of PKC have been found in a variety of cellular compartments including plasma membrane, cytosol, cytoskeletal elements, Golgi apparatus, the perinuclear and the nuclear fractions (Mochly-Rosen, et al., 1991). Interestingly, Mochly-Rosen et al. (1991) reported that, like various PKC isoforms, substrates for these kinases are also present in different subcellular compartments, thus supporting the possibility of a differential role of PKC isoforms in the regulation of different physiological processes.

Another possibile interpretation of the dissimilar steroidogenic responses of granulosa cells to OAG and TPA may be related to the action (s) of PKC activators on Ca^{2+} metabolism. Ca^{2+} is important for the hormonal regulation of granulosa cell steroidogenesis. It plays a pivotal role in the regulation of 3β-hydroxysteroid dehydrogenase (metabolism of P_5 to P), without a significant effect on the other steroidogenic enzymes (Tsang and Carnegie, 1983; Veldhuis et al., 1984; Asem et al.,

1987, Leung and Wang, 1989). In the present study, calcium ionophore A23187 significantly increased the secretion of P and the conversion of exogenous P_5 to progestins during 24 h cultures, whereas OAG or TPA exerted different effects on A23187-stimulated P secretion. TPA alone inhibited the metabolism of P_5 to P and reduced A23187-stimulated steroid secretion to a level characteristic of the presence of phorbol ester only. In contrast, OAG markedly increased basal conversion of P_5 to progestins but in the presence of the Ca^{2+} ionophore, did not enhance steroid secretion beyond that seen with A23187 alone. PKC has been suggested to modulate several membrane proteins related to channels, pumps and ion exchange carriers of Ca^{2+}. Investigations with various nonsteroidogenic cells have indicated that TPA stimulates Ca^{2+} extrusion. It can facilitate Ca^{2+} efflux (Pollock et al., 1987; Smallwood et al., 1988; Furukawa et al., 1989), inhibit Ca^{2+} influx (Sagi-Eisenberg et al., 1985; He et al., 1988; Berggren et al., 1989) and/or mobilization from intracellular stores (Owen, 1988; He et al., 1988; Willems et al., 1989) resulting in decreased intracellular Ca^{2+} concentration. Unlike TPA, OAG has been suggested to act as a Ca^{2+} ionophore via its metabolite, phosphatidic acid, which increases intracellular Ca^{2+} concentration by either mediating the inward movement of this ion (Tyson et al., 1976; Putney et al., 1980) or by stimulating IP_3 production and the release of Ca^{2+} from intracellular stores (Murayama and Ui, 1985; Moolenaar et al., 1986).

In this context, the decrease in P secretion observed in the presence of A23187 and TPA (compared to steroid level in the presence of calcium ionophore alone), could have been due to the extrusion of Ca^{2+} immediately after its mobilization into the cytosol. It is possible that a Ca^{2+}-transporting ATPase, believed to be a target for PKC action (Berggren at. al, 1989; Willems et al., 1989; Furukawa et al., 1989), actively participated in the regulation of Ca^{2+} efflux. The reason for the observed attenuation of the stimulatory action of OAG in the presence of A23187 is not clear. However, since Ca^{2+} is believed to have a biphasic effect on steroid secretion, with stimulation at lower

concentrations and inhibition at high concentrations of the ion, it is conceivable that the surplus of Ca^{2+} brought into the cell by A23187 in addition to that by OAG or phosphatidic acid could have partially suppressed the increase in P secretion evoked by OAG alone. A23187 has been shown to stimulate basal secretion of P, but to reduce the production of this steroid induced by high but not low concentrations of FSH and cAMP (Tsang and Carnegie, 1984; Leung et al., 1988). Since OAG increases steroidogenesis in rat granulosa cells in the presence and absence of an agonist, the mechanism of action of OAG might in part be common to that of FSH and could have been involved in the inhibition by A23187 in a manner similar to that of gonadotropin.

Another possible reason for the diversity of OAG and TPA actions on FSH-stimulated steroidogenesis could be their differential effects on signalling systems other than those of Ca^{2+} and PKC. Exogenous diacylglycerols and tumor promoting phorbol esters have been shown to promote the hydrolysis of membrane phosphatidylcholine (PC) through the activation of phospholipases of the A_2 (PLA_2), C (PLC) and D (PLD) types in many cells (Oishi et al., 1988; Exton, 1990). While the hydrolysis of PC by PLA_2 appears to be an important source of AA (subsequently metabolized to eicosanoids), the breakdown of PC by PLC and PLD yields diacylglycerols and phosphatidic acid, respectively (Exton, 1990). Analysis of the molecular species of PC and diacylglycerols following prolonged agonist stimulation has demonstrated that PC-derived diacylglycerols are effective in activating PKC in vitro (Go et al., 1987). Activation of PKC by the products of PC hydrolysis seems to last longer than that from PIP_2 breakdown (Matozaki and Williams, 1989) and may be due to a to much greater cellular content of PC as compared to PIP_2. Recent findings suggest that diacylglycerols derived from PC have different cellular effects than those released during PIP_2 breakdown (Exton, 1990). In this context, it is of interest that exogenous diacylglycerols (DiC_8 and OAG) but not TPA stimulated the metabolism of phosphatidylcholine in GH_3 cells in vitro (Kolesnik and Paley, 1987).

AA, released during the activation of PLC and/or PLA_2, has been shown to stimulate basal and TPA induced P production by granulosa cells in vitro (Wang and Leung, 1988). In addition, exogenous AA partially antagonized the inhibitory effects of GnRH on FSH-stimulated P production and of TPA on cholera toxin-induced production of the hormone (Wang and Leung, 1989). These results are similar to the present observations regarding cotreatment of granulosa cells with OAG and TPA, since progestin secretion was increased to a significantly greater extent in the presence of both PKC activators than in the presence of OAG or TPA alone. The inhibition of FSH-stimulated steroidogenesis by TPA in a 24 h culture period was reduced to the control level when OAG was also added to the medium, implicating a possible action of OAG via AA (released from diacylglycerol metabolism and/or hydrolysis of membrane PC). Leung and co-workers reported that GnRH caused a rapid release of AA from membrane phospholipids in rat granulosa cells (Minegishi and Leung, 1985; Wang and Leung, 1988) and postulated that AA mediates predominantly the stimulatory but not the inhibitory action of GnRH on ovarian steroidogenesis (Leung and Wang, 1989).

It is tempting to speculate that the effects of continual incubation with exogenous diacylglycerols in the present study may in part be due to activation of PC breakdown, thus mimicking the physiological action of some intra-ovarian factors. Certain growth factors (EGF, PDGF) have been shown to stimulate the hydrolysis of PC and to sustain elevated levels of diacylglycerols for several hours (Wright et al., 1988; Farese et al., 1989). EGF increased $P_{-450scc}$ mRNA concentration in swine (Urban et al., 1990) and enhanced basal (Bendeli and Dorrington, 1988), FSH- and IGF-stimulated 3β-HSD activity in rat granulosa cells (Bendell and Dorrington, 1988). EGF also augmented FSH-induced P_5, P and 20α-OH-P production in granulosa cells from immature hypophysectomized rats (Jones et al., 1982) and increased gonadotropin- and cAMP-stimulated P production in luteinized human granulosa cells (Tapanainen et al., 1987; Tippet et al., 1988) and in immature mouse ovaries in vitro (Vorob'eva et al., 1991). In

the present study OAG potentiated FSH- and $(Bu)_2$cAMP-stimulated P_5 and progestin secretion, thus resembling the effect of EGF on human (Tippet et al., 1988) and rat (Jones et al., 1982) granulosa cells. Interestingly, the magnitude of increase by OAG of the gonadotropin and cAMP effects reported by Tippet et al. (1988) was similar to that observed in the present experiments.

TPA and OAG appear to share, in part, a common pathway in their stimulation of basal steroids secretion, but to act differently in the regulation of FSH-induced steroidogenesis in rat granulosa cells in vitro. While the effects of TPA resemble those of GnRH, an intra-ovarian regulator known to stimulate basal secretion of P and to reduce gonadotropin-stimulated steroid and cAMP production in rat granulosa cells (Hsueh and Jones, 1981; Kawai and Clark, 1985; Wang and Leung, 1987; Leung et al., 1988, 1989), OAG may mimic the physiologic action of EGF and/or perhaps only the stimulatory aspect of GnRH function in these cells. However, it appears that the mechanisms involved in the regulation of rat granulosa cell steroidogenesis in the presence of diacylglycerols and TPA include PKC activation, since down-regulation of the enzyme by pretreatment with TPA prevented any subsequent stimulation by the PKC activators. Whether the different isoforms of PKC are responsible for divergent effects of OAG and TPA on FSH-stimulated P_5 and P secretion remains to be determined.

In summary, based on previously published information and results from the present study, the following working model is proposed for future investigation. The DG/PKC pathway is involved in the control of cholesterol delivery to the mitochondria and in the regulation of the activities of steroidogenic enzymes, catalyzing each step in the metabolism of cholesterol to progestins (cholesterol $\rightarrow P_5 \rightarrow P \leftrightarrow 20\alpha$-OH-P) in rat granulosa cells (Fig. 45). Moreover, I propose a multiple role for DG/PKC pathway in FSH-stimulated steroidogenesis in rat granulosa cells: one in which DG/PKC functions as a **mediator** of FSH action (in addition to the involvement of the cAMP/PKA pathway in FSH - induced steroid secretion in granulosa cells) (Fig. 46); another with DG/PKC as a

mediator of different agonist which modulate the FSH-induced cAMP production and action, and as a regulator of intracellular Ca^{2+} mobilization in granulosa cells (Fig. 47).

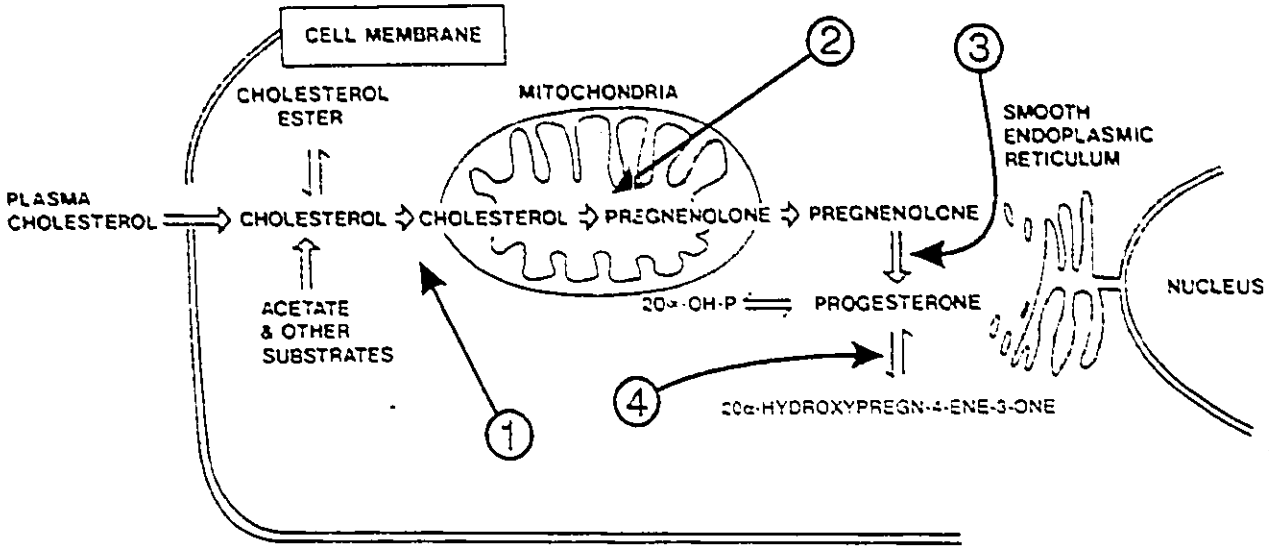

Figure 45. Proposed sites of PKC action on granulosa cell steroidogenesis in vitro.

1.- Sterol carrier protein 2 (SCP_2).

2.- Cholesterol side-chain cleavage cytochrome P_{450} (P_{450scc}).

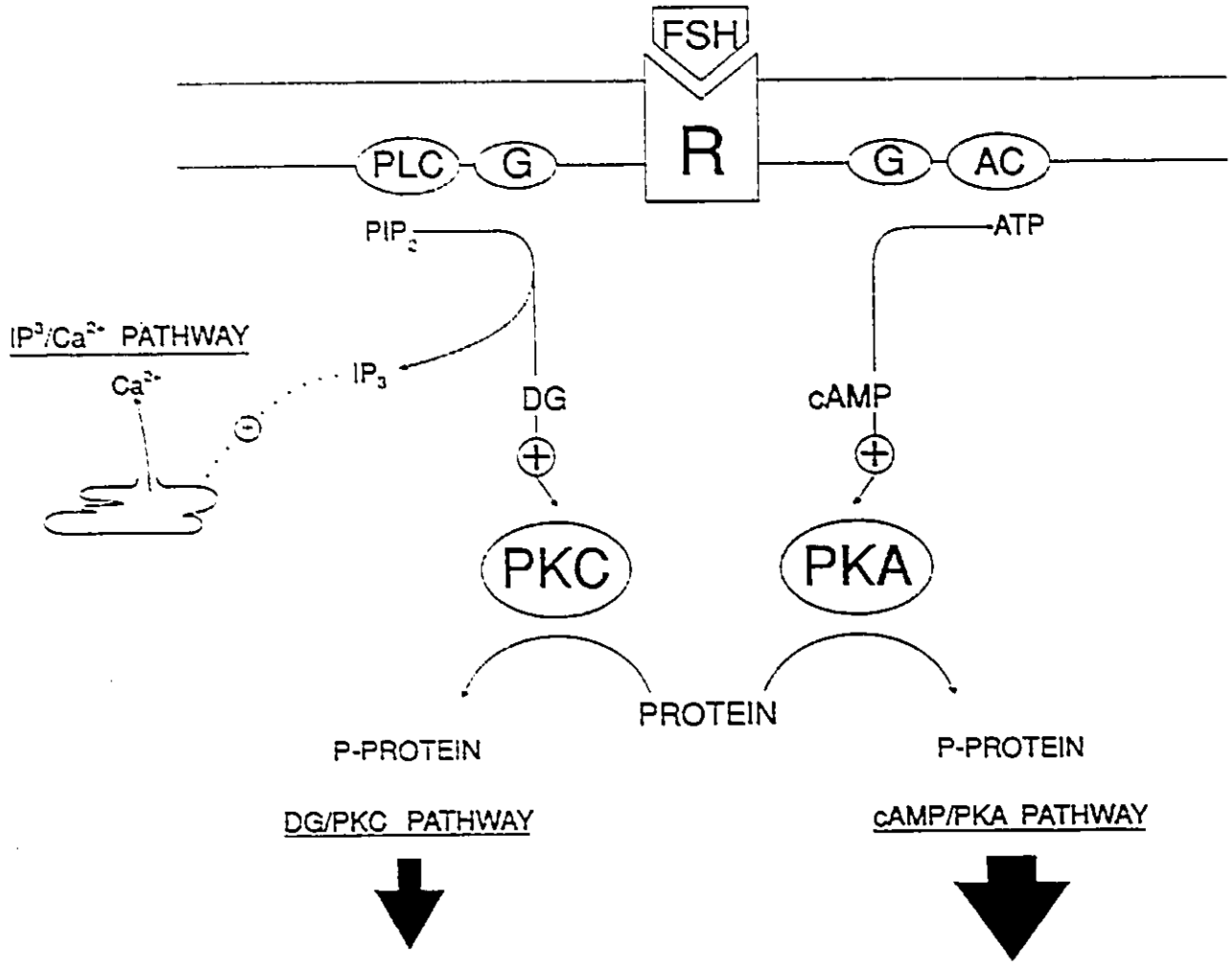

Figure 46 Proposed mediatory role of DG/PKC pathway for FSH signalling system in granulosa cells. (Modified after Davis, 1991)

Binding of FSH to cell surface receptor (R) activates, via respective G proteins, adenylyl cyclase (AC) and phospholipase C (PLC). AC hydrolyzes ATP to cAMP. The latter activates protein kinase A (PKA) which phosphorylates and activates proteins; PLC hydrolyzes phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DG) and inositol trisphosphate (IP_3). IP_3 mobilizes Ca^{2+} from intracellular stores; DG activates protein kinase C (PKC) which phosphorylates and activates respective proteins. cAMP/PKA pathway appears to exert primary control over steroid secretion in granulosa cells.

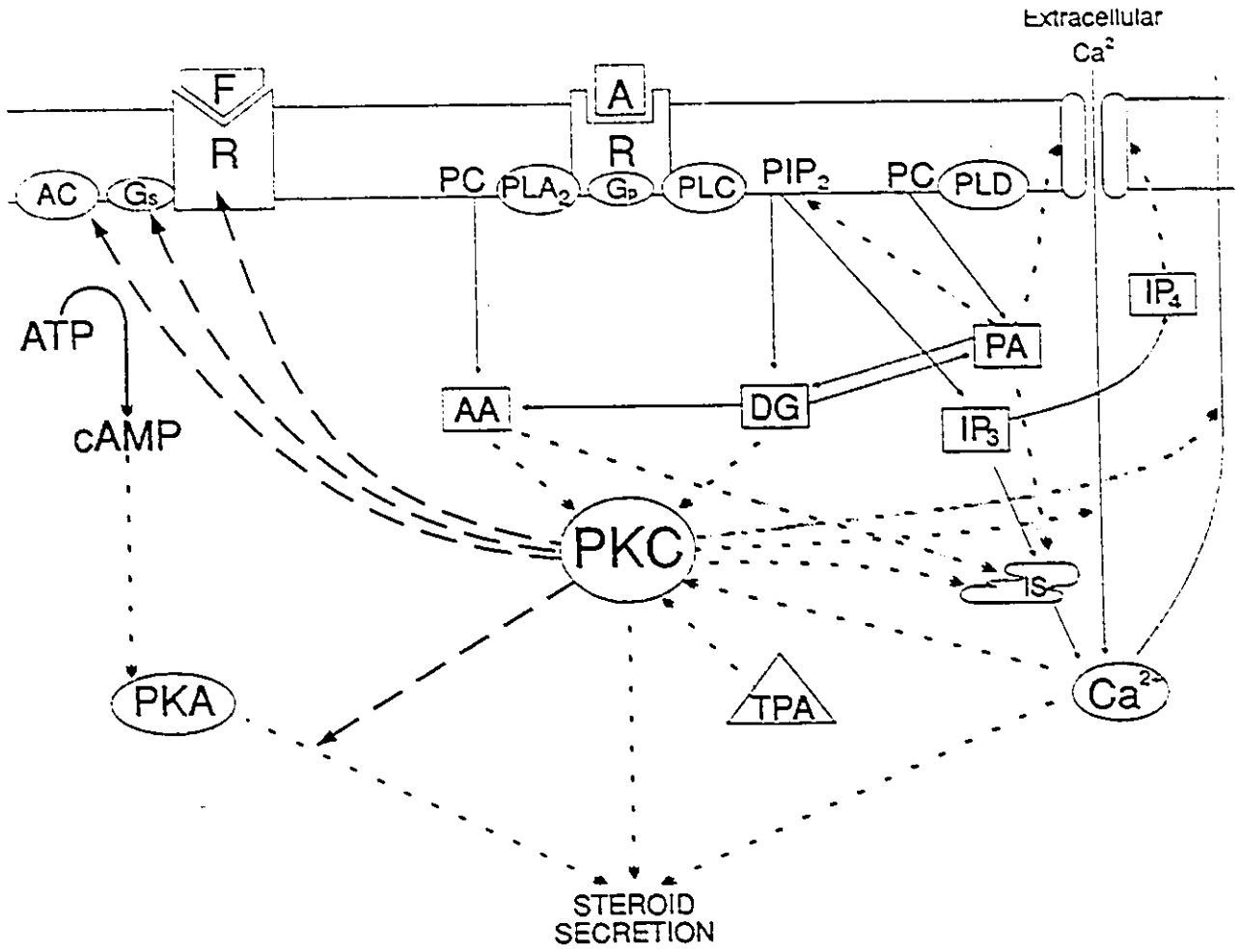

Figure 47 Proposed modulatory role of DG/PKC pathway in granulosa cells.

Binding of agonist (A) to cell surface receptor (R) activates, via G protein (G_p), phospholipase C (PLC). The latter hydrolyzes phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DG) and inositol trisphosphate (IP_3). Another source of DG can be phosphatidic acid (PA) released during hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD). DG activates protein kinase C (PKC). The enzyme is also activated by arachidonic acid [AA; metabolized from DG or alternatively from PC during phospholipase A_2 (PLA_2) hydrolysis of PC] and by increased Ca^{2+} levels. Ca^{2+} is mobilized by IP_3 from intracellular stores and IP_4 from extracellular Ca^{2+} milieu, via Ca^{2+} channels. Activated PKC modulates FSH (F) - receptor interaction, activation of G protein (G_s) and/or activation of adenylyl cyclase (AC). By these means PKC regulates cAMP formation and activation of protein kinase A (PKA) which phosphorylates and activates proteins involved in steroidogenesis. PKC influences also steroid production at sites distal to cAMP formation. Activators of PKC (DG, AA and TPA) are believed to modulate intracellular Ca^{2+} levels by regulating: IP_3 formation (PA), release of Ca^{2+} from intracellular stores (IS; AA, TPA) or Ca^{2+} influx/efflux via Ca^{2+} channels (PA, TPA). Arrows depict metabolism (————), modulation (— — —) and regulation (– – – –).

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