ACTION OF LUTEINIZING HORMONE-RELEASING HORMONE IN RAT OVARIAN CELLS: HORMONE PRODUCTION AND SIGNAL TRANSDUCTION

by

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Abstract

The present study was conducted to investigate the hypothesis that membrane phosphoinositide breakdown may participate in the actions of luteinizing hormone-releasing hormone (LHRH) on hormone production in the rat ovary.

In granulosa cells prelabeled with [^{3}H]-arachidonic acid or [^{3}H]-inositol, treatment with LHRH increased the accumulation of radiolabeled inositol lipids, diacylglycerol and free arachidonic acid, but luteinizing hormone (LH) or cholera toxin did not exert the same effect. Activation of protein kinase C by the phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate (TPA) had a stimulatory action on membrane phosphoinositide breakdown. In addition, TPA did not alter arachidonic acid release but potentiated the A23187 stimulated liberation of arachidonic acid.

Changes in the cytosolic free calcium ion concentrations, [Ca^{2+}]i, induced by LHRH were studied in individual cells using fura-2 microspectrofluorimetry. The resting [Ca^{2+}]i was 96.7 ± 2.9 nM (n= 115). The alterations in [Ca^{2+}]i induced by LHRH were transient and returned to resting levels within 84±3 second (n=64). A potent LHRH antagonist completely blocked the effect of LHRH on [Ca^{2+}]i. Some cells responded to LHRH alone, whereas others responded to angiotensin II, suggesting that there are different subpopulations of granulosa cells. Sustained perifusion of LHRH resulted in a desensitization of the [Ca^{2+}]i response to LHRH but not to the calcium ionophore A23187. LHRH treatment accelerated [Ca^{2+}]i depletion in

granulosa cells perifused with Ca^{2+} free medium, indicating the involvement of intracellular Ca^{2+} pool(s) in $[Ca^{2+}]i$ changes induced by LHRH.

The complex interactions between the signal transduction pathways involved in the regulation of progesterone and prostaglandin E_2 were also examined. LHRH increased basal progesterone level (5 and 24h culture) and attenuated progesterone production induced by follicle-stimulating hormone (FSH) or cholera toxin (24h). On the other hand, both basal and FSH or cholera toxin stimulated prostaglandin E_2 formation were increased by LHRH (5 and 24h). A23187, TPA and melittin (an activator of phospholipase A_2) were used to examine the roles of Ca^{2+}, protein kinase C and free arachidonic acid, respectively, in LHRH action. Melittin stimulated basal progesterone and prostaglandin E_2 production, and enhanced the stimulation of prostaglandin E_2 by LHRH, A23187 and TPA, indicating that LHRH alters cyclooxygenase activity. A23187 or TPA attenuated the formation of progesterone induced by FSH or cholera toxin (5 and 24h). In contrast, A23187 and TPA augmented cholera toxin or FSH induced prostaglandin E_2 formation. The stimulatory effects of A23187 and TPA on prostaglandin E_2 were synergistic, whether or not FSH or cholera toxin was present during the incubation.

The role of arachidonic acid in the action of LHRH was further investigated. Arachidonic acid enhanced progesterone production in a dose dependent manner and potentiated TPA induced progesterone production. The stimulatory effect of arachidonic acid was blocked by nordihydroguaiaretic acid,

whereas monohydroxyeicosatetraenoic acids and hydroperoxyeicosatetraenoic acid mimicked the effect of arachidonic acid, suggesting the involvement of lipoxygenase metabolites in LHRH action. In addition, arachidonic acid partially reversed the inhibitory action of LHRH and TPA on FSH induced progesterone production. Although arachidonic acid, TPA and LHRH stimulated progesterone production, arachidonic acid only slightly elevated 20-alpha-hydroxy-progesterone production as compared to that induced by LHRH and TPA. These results suggest that arachidonic acid or its metabolites have a stimulatory role in the action of LHRH on the de novo synthesis of ovarian steroid hormones.

Collectively, these findings support the hypothesis that the actions of LHRH or LHRH like peptides on ovarian hormone production are mediated by multiple second messengers involving Ca^{2+}, protein kinase C and arachidonic acid metabolites.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AA	arachidonic acid
GABA	gamma-aminobutyric acid
°C	degree Celsius
$[Ca^{2+}]i$	intracellular calcium ion concentration
cAMP	3' 5'-cyclic adenosine monophosphate
CT	cholera toxin
DG	1,2-diacylglycerol
DPM	disintegration per minute
ER	endoplasmic reticulum
EBSS	Earl's Balanced Salt Solution
FBS	fetal bovine serum
FSH	follicle stimulating hormone
G	guanine nucleotide regulatory protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
hCG	human chorionic gonadotrophin
HDL	high density lipoprotein
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
20-alpha-HSD	20-alpha-hydroxysteroid dehydrogenase
3-beta-HSD	3-beta-hydroxysteroid dehydrogenase
17-beta-HSD	17-beta-hydroxysteroid dehydrogenase
IP_3	inositol 1,4,5-trisphosphate
IU	international unit

LDL	low density lipoprotein
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LT	leukotriene
LX	lipoxin
min	minute
M	molar
NADPH	nicotinamide adenine dinucleotide phosphate
NDGA	nordihydroguaiaretic acid
20-alpha-OH-P	20-alpha-hydroxypregn-4-en-3-one
P	statistical probability
P_4	progesterone
PG	prostaglandin
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-phosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMSG	pregnant mare's serum gonadotrophin
PRL	prolactin
RIA	radio-immunoassay
SCC	side-chain cleavage
sec	second
SE	standard error
TPA	12-O-tetradecanolyphorbol-13-acetate
TX	thromboxane

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Chapter 1. General Introduction

I. Ovary

A. Introduction

The function of the ovary is to produce mature eggs and secrete ovarian hormones. The latter exert a range of effects including regulation of the reproductive system, secondary sex characters, the mating behavior of some species, pituitary gonadotropin release and metabolic effects. The gametogenic and endocrine functions of the ovary in the female are cyclic processes exhibiting regular peaks of activity during the life of the individual, and may be regarded as periodic preparations for fertilization and pregnancy. The periodicity is called the estrous cycle in subprimate species and the menstrual cycle in primates. The cyclical changes occur as a result of complex integrated activity of the hypothalamus, pituitary and ovaries. The most important hormone signals of this system are luteinizing hormone-releasing hormone (LHRH) from the hypothalamus, follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland and the ovarian steroid hormones such as androgens, estrogens and progesterone (P_4). The gonadotroph cells of pituitary synthesize and secrete LH and FSH in response to LHRH. LH and FSH arrive at the ovary via the circulatory system. FSH causes ovarian follicular growth, while the LH surge induces ovulation and regulates corpus luteum formation and function. Both FSH

and LH are necessary to stimulate ovarian steroidogenesis. Ovarian steroid hormones, especially estradiol and P_4, in turn, regulate FSH, LH and LHRH release by either a positive or negative feedback mechanism depending on the stage of the estrous or menstrual cycle. Recently, it has been shown that a family of peptides known as inhibin regulates FSH release selectively, and that the production of these peptides is controlled by FSH. The inhibins thus represent an additional closed feedback loop between the pituitary and ovary to regulate reproductive functions (Rivier et al., 1986). Other local regulatory factors such as prostaglandins and LHRH-like peptides may also be involved in the regulation of reproductive functions.

B. Histology

The ovaries are paired organs situated on either side of the uterus. Each ovary is covered by a continuous mesothelium composed of a single layer of cuboidal epithelium. The ovary is roughly divided into a peripheral cortex and a medulla. The cortex contains numerous ovarian follicles that consist of a primary oocyte enveloped by a single layer of spindle-shaped granulosa cells in various stages of development and a dense connective tissue stroma. The medulla is small compared to the cortex, and its connective tissue is loosely arranged. Embedded within the loose connective tissue of the medulla are nerves, lymph vessels and many large blood vessels. Small blood vessels extend from the medulla into the cortex.

Cortical stroma consists of at least three types of cells: connective tissue cells performing the customary support functions, contractile cells scattered in the cortical stroma and in the walls of preovulatory follicles, and closely packed spindle-shaped interstitial cells.

Four major classes of interstitial cells have been identified: 1) primary interstitial; 2) theca interstitial; 3) secondary interstitial; and 4) hilus interstitial cells. Although these cells are located in the loose connective tissue of both the cortex and medulla, all arise from a population of unspecialized mesenchymal cells in the stroma compartment. The principal function of the interstitial cells is to synthesize and secrete steroids, most notably androstenedione and testosterone.

It appears that granulosa cells are derived mainly from certain cells within the intraovarian rete ovarii which resemble granulosa cells in terms of their organelles and microfilaments (Byskov, 1978; Byskov and Rasmussen, 1973). The differentiation of granulosa cells is not uniform in a given follicle. As the antral follicle develops, these cells become organized into morphologically distinguishable regions with specialized functions. At least three different populations of granulosa cells can be distinguished. The antral granulosa cells are closer to the antral cavity, while the cumulus cells surround the oocyte. Cumulus cells physically support the oocyte within the follicle and provide nutrients for oocyte growth. They also probably exchange signals with the oocyte

for the coordinated maturation of the follicle and the oocyte. The majority of granulosa cells are mural or parietal granulosa cells lining the follicular cavity.

C. Life cycle of the ovarian follicle

The primordial follicles are present before birth. The oocyte and associated spindle-shaped cells are separated from the surrounding stroma by the basal membrane. One of the basic events within the ovary is follicular growth, an irreversible process, which results in ovulation or atresia. At the onset of puberty, primordial follicles mature into primary follicles, which are subject to intra-ovarian controls (Peters et al., 1975). Follicular maturation is initiated when the spindle-shaped granulosa cell precursors differentiate into a single layer of cuboidal cells that then begin to divide (Van Wagenen and Simpson, 1965). The oocyte increases in size and the granulosa cells proliferate mitotically. Granulosa cells synthesize and secrete mucopolysaccharides, which give rise to the zona pellucida that surrounds the oocyte. After the granulosa cells begin to proliferate in the primary follicles, the follicle becomes encapsulated by distinct layers of theca cells, the theca interna and the theca externa. The theca interna is separated from the granulosa layer by the basement membrane. Blood vessels and lymphatics penetrate the theca externa but do not penetrate the basement membrane thus granulosa cells are without direct blood supply until after ovulation. As the follicle grows, the granulosa cells increase

in number and size. Follicular fluid accumulates within the follicle and coalesces to form a single cavity, the antrum. Antral formation transforms the primary follicle into a Graafian follicle. Within this is the cumulus oophorus, an accumulation of granulosa cells containing the oocyte. This oocyte is liberated when the mature follicle ruptures following the LH surge in a process called ovulation.

Following ovulation, the corpus luteum is formed from both the granulosa and theca interna cells. The basement membrane breaks down, and capillaries and fibroblasts from the theca interna invade the cavity of the ruptured follicle. The granulosa cells do not divide after ovulation, but they increase in volume and undergo morphologic changes with an increase in masses of lipid droplets, smooth endoplasmic reticulum, and mitochondria. These changes are referred to as luteinization. Since the cells of the corpus luteum are derived from both granulosa and theca cells, the corpus luteum consists of two types of steroidogenic luteal cells, which are morphologically distinct, the large luteal cells and the small luteal cells. These cells, together with the surrounding theca cells, capillaries and blood vessels form the corpus luteum, a temporary endocrine gland that secretes large amounts of steroid hormones.

II. Synthesis of sex steroid hormones and prostaglandins

A. Synthesis of sex steroids

Ovaries have the capacity to synthesize all three classes of sex steroid hormones from their common precursor, cholesterol (Fig. 1). Cholesterol from both low-density lipoproteins (LDL) and high-density lipoproteins (HDL) has been demonstrated to serve as precursor for steroidogenesis in the ovarian follicle (Gwynne and Strauss, 1982). While HDL appears to be the major precursor in rodents, cholesterol from LDL is the major precursor in other species. Cellular cholesterol may be derived from plasma lipoprotein, from cytoplasmic lipid droplets or synthesized de novo in ovarian cells (Strauss et al., 1981). Uptake of lipoprotein from plasma is regulated by the availability of serum lipoproteins and the lipoprotein receptor-dependent uptake system. Cholesterol can be stored in the cells as esters of long-chain fatty acid and this process is regulated by the relative activities of cholesterol synthetase and cholesterol esterase. Additionally, de novo synthesis of cholesterol is dependent on the activities of the rate-limiting 3-hydroxy-methylglutaryl coenzyme A reductase (Brown et al., 1981).

Granulosa cells are the cellular source of the two most important ovarian steroids, estradiol and P_4. The first step in the conversion of cholesterol to steroids is believed to be rate limiting in steroidogenesis, and involves the cleavage of the cholesterol side-chain by the side-chain cleavage P-450

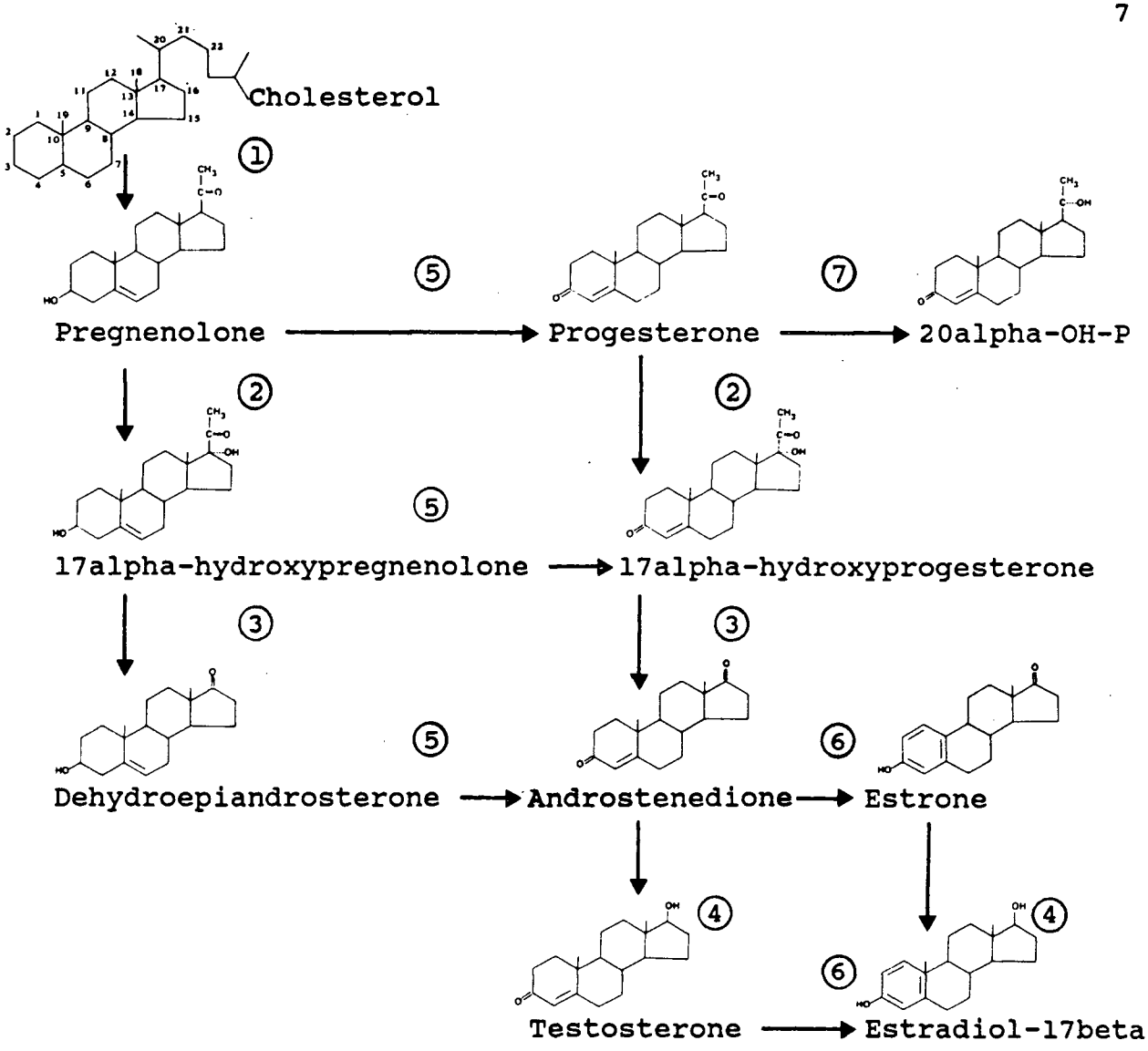

Fig. 1. The principal biosythetic pathway in the ovary for production of the progestins, androgens and estrogens.
1: cholesterol side-chain cleavage P_{450}

2: 17-alpha-hydroxylase

3: $C_{17,\ 20}$-lyase

4: 17-beta-hydroxysteroid dehydrogenase

5: 3-beta-hydroxysteroid dehydrogenase/Δ^5,Δ^4 isomerase.

6: Aromatase

7: 20-alpha-hydroxysteroid dehydrogenase

20alpha-OH-P: 20alpha-hydroxypregn-4-en-3-one

enzyme (SCC) resulting in the C_{21} compound, pregnenolone. SCC, including cholesterol 22-hydroxylase, cholesterol 20-alpha-hydroxylase and $C_{20,\ 22}$-lyase, are located in the inner mitochondrial membrane.

Pregnenolone is the key steroidogenic intermediate common to all classes of steroid hormones produced by the follicles. Both granulosa and theca cells convert pregnenolone to P_4, but granulosa cells are more active in this regard (Bjersing, 1967). Pregnenolone is converted to P_4 by a complex of two enzymes 3-beta-hydroxysteroid dehydrogenase (3-beta-HSD) and an isomerase (Samuels et al., 1951; Cheatum et al., 1966). Both enzymes requiring nicotinamide adenine dinucleotide (NAD) as a cofactor are located in the microsomal fraction, although 3-beta-HSD may also be present in the mitochondria of the ovary (Sulimovici and Boyd 1969; Haksar and Romanoff, 1971; Dimino and Campbell, 1976). Since isomerase activity appears to be in excess (Philpott and Peron, 1971), the production of P_4 from pregnenolone is mainly regulated by 3-beta-HSD.

The rate-limiting step in the biosynthesis of androgens in the follicle is that catalyzed by the 17-alpha-hydroxylase/ $C_{17,20}$-lyase enzyme complex which is located in the microsomal fraction of the cells and which requires nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen for its action. Hydroxylation at the C_{17} position is essential before the side chain is cleaved from the C_{21} steroids (progestins) to form C_{19} steroids (androgens). The reaction can utilize both pregnenolone and P_4 as substrates resulting in

dehydroepiandrosterone or androstenedione, respectively. This enzymatic step which is under the control of hormones and feedback regulation by the end products of steroidogenesis, is one of the key points for the physiologic control of follicular steroid secretion.

In contrast to the neighboring theca cells, the granulosa cells contain very low levels of the enzymes, 17-alpha-hydroxylase and $C_{17,20}$-lyase, which mediate the conversion of progestins to androgens (Short, 1962; Bjersing and Carstensen, 1967). The deficiency of these enzymes in granulosa cells indicates that both granulosa cells and theca cells participate in androgen and estrogen biosynthesis.

The conversion of androstenedione and testosterone to estrone and estradiol-17-beta is catalyzed by an enzyme complex, referred to as aromatase, located in the membranes of the agranular endoplasmic reticulum of several ovarian cell types. The reaction requires NADPH, and three moles of oxygens. Two of these are involved in two consequent hydroxylation at C-19, and the overall reaction involves a third hydroxylation, but the exact site of this is not yet clear (Kantsky and Hagerman, 1980; Brodie et al., 1976).

The secretion of P_4 by ovarian cells is modulated by changes in the conversion of P_4 to its metabolites. The main route of P_4 breakdown is mediated by 20-alpha-hydroxysteroid dehydrogenase (20-alpha-HSD), located in the cytosol portion of ovarian cells utilizing NADPH as a hydrogen donor, which reversibly converts P_4 to its inactive metabolite, 20alpha-

hydroxy-pregn-4-en-3-one (20-alpha-OH-P). 20-alpha-OH-P is considerably less active as a progestational agent than its precursor P_4. It has been suggested that the activity of 20alpha-OH-P may play a significant role in determining the amount of C_{21}-substrate available for conversion to androgens in follicular cells, since 20alpha-reduced steroids are poor substrates for $C_{17,20}$-lyase (Goldring and Orly, 1985).

B. Synthesis of Prostaglandins and Leukotrienes

Prostaglandins (PGs), which were first discovered by Von Euler in the 1930s as a biologically active component of human seminal fluid, are also important secretory products of the ovarian cells and the secretion of prostaglandins may be under hormonal control (Triebwasser et al., 1978; Clark et al., 1978). The precursor for PGs synthesis is arachidonic acid (AA), which is a C 20:4 polyunsaturated fatty acid. AA in mammalian cells is normally esterified almost exclusively in the 2-acyl position to glycerol in the phospholipids of the cell membrane and is released through a phospholipase-catalyzed reaction. The concentration of free AA in cells is less than 10^{-6}M, and the free acid level in a tissue represents a balance between the liberation of the acid by hydrolysis and its re-esterification. Free AA can undergo two oxidative pathways of metabolism as outlined in Figure 2. The cyclooxygenase pathway leads to the formation of the endoperoxide intermediate prostaglandin H_2, which is then converted by the action of isomerases to a number of biologically active molecules,

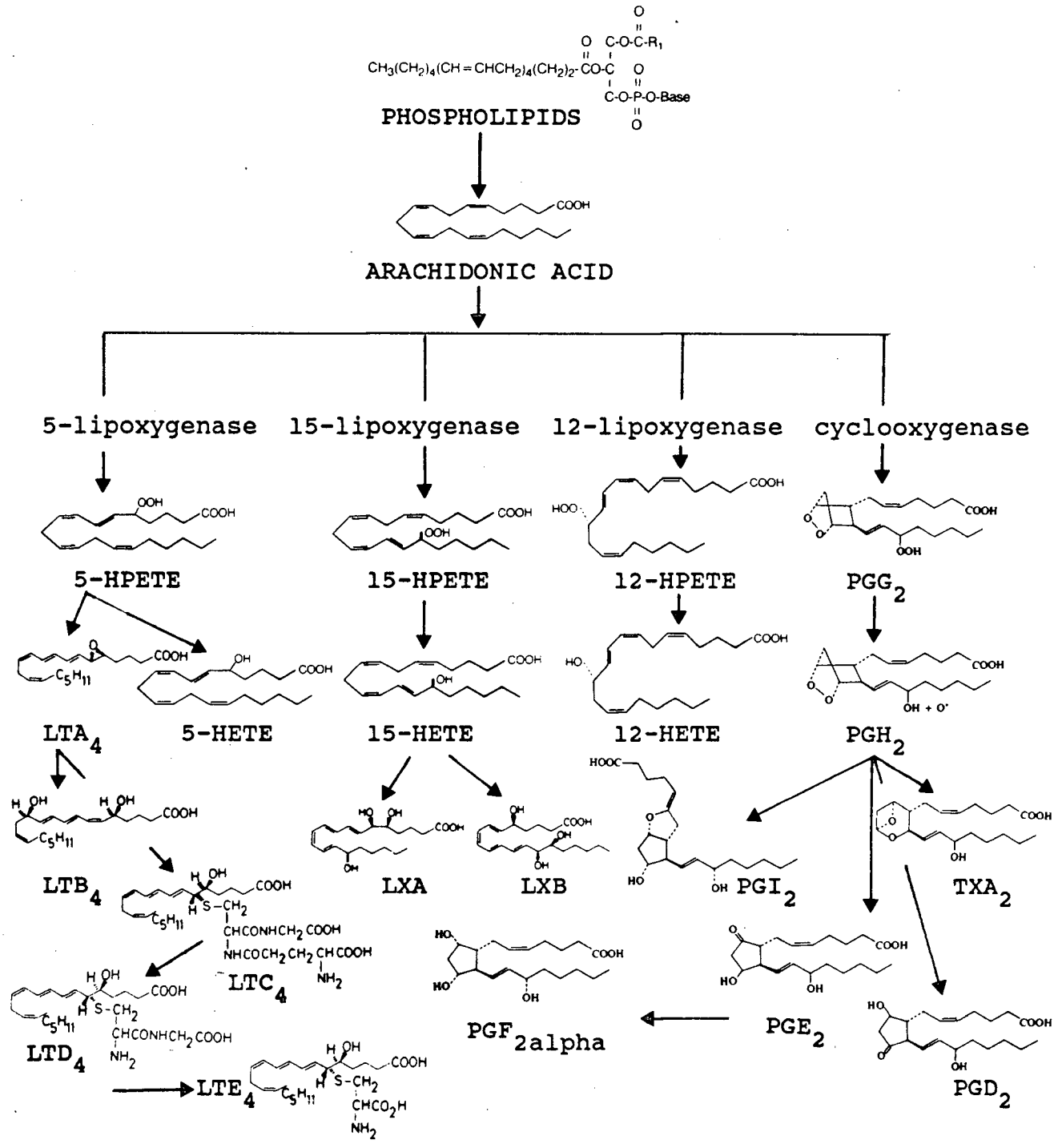

Fig. 2. Key pathways in arachidonic acid metabolites.
PG: prostaglandin
LT: leukotriene
LX: lipoxin
HETE: hydroxyeicosatetraenoic acid
HPETE: hydroperoxyeicosatetraenoic acid

prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin F_{2alpha} (PGF_{2alpha}), prostaglandin I_2 (PGI_2) and thromboxane A_2 (TXA_2). The letters following the abbreviation PG indicate the nature and position of the oxygen-containing substituents present in the cyclopentane ring. The 2-series PGs are formed from AA, and the 1-series and 3-series PGs are synthesized from 8,11,14-eicosatrienoic and 5,8,11,14,17-eicosapentaenoic acid, respectively. An alternative pathway for the oxygenation of AA is provided by lipoxygenase enzymes. The products of the lipoxygenase enzymes are hydroperoxyeicosatetraenoic acids (HPETEs) which can then be converted into hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs), and lipoxins (Fig. 2).

Rat ovarian and follicular homogenates possess lipoxygenase activity that increases after in vivo administration of human chorionic gonadotropin (hCG) (Reich, 1985). The induction by hCG of PGs is demonstrated to occur both in granulosa cell and theca cells of preovulatory follicles (Hedin et al., 1987). The activity of cyclooxygenase can be inhibited by nonsteroidal anti-inflammatory drugs such as aspirin and indomethacin (Flower and Vane, 1974). Inhibition of PG cyclooxygenase effectively blocks the synthesis of all cyclooxygenase series. Lipoxygenase activity can be inhibited by compounds such as nordihydroguaiaretic acid (NDGA) (Salari et al., 1984).

III. Regulation of ovarian hormone synthesis

A. Role of gonadotropins

Ovarian follicle growth and steroid hormone production are mainly under the control of two gonadotropin hormones LH and FSH. LH and FSH are synthesized and stored in the anterior pituitary and are released in response to luteinizing hormone-releasing hormone (LHRH). LHRH is a decapeptide (pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) found in the hypothalamus of all mammalian species so far studied. LHRH is secreted into the hypophyseal portal system in a pulsatile fashion and is transported along the pituitary stalk to the pituitary. In the pituitary, LHRH controls the synthesis and secretion of gonadotropins by a receptor dependent mechanism. Gonadotropins are likewise released in a pulsatile pattern. The pulsatile release and cyclic variation in the circulating concentrations of gonadotropins control ovarian functions by altering the sensitivity of ovarian cells through increasing and decreasing receptor formation and activities of cellular enzymes.

Although the initiation of primordial follicle growth occurs independently of pituitary gonadotropins, once reaching the primary follicle stage further growth and maturation of the follicle becomes completely dependent on LH and FSH.

FSH induces ovarian follicle maturation and is responsible for the development of granulosa cell responsiveness to several other hormones. FSH interacts with ovarian cells through specific plasma membrane receptors. In

the female, FSH binds only to the granulosa cells of the ovarian follicles. FSH regulates granulosa cell progestin biosynthesis by modulating the activities of various steroidogenic enzymes, SCC, 3-beta-HSD and 20-alpha-HSD (Toaff et al., 1983). FSH also induces aromatase. LH stimulates preovulatory follicle growth, induces ovulation, and regulates corpus luteum function. The major site of action of LH on P_4 biosynthesis is the conversion of cholesterol to pregnenolone, although 3-beta-HSD is also stimulated (Armstrong et al., 1970; Madej, 1980). After the FSH induction of LH receptors in granulosa cells, these cells are capable of responding to LH in the maintenance of aromatase activity (Wang et al., 1981; Dorrington and Armstrong, 1979). The steroidogenic action of LH on theca cells apparently increases the activities of 17-alpha-hydroxylase and $C_{17,\ 20}$-lyase in ovaries (Fukuda et al., 1979; Bogovich and Richards, 1982).

Previous studies have demonstrated a "two cell-type, two gonadotropin theory" (Fig. 3). There are principal cell types involved in follicular steroidogenesis: (1) LH-responsive secretory cells: comprising the theca interna cells of the follicular envelope and the interstitial cells of ovarian stroma, and (2) FSH-responsive cells which are granulosa cells. According to this model, theca interna cells are stimulated by LH to produce androgen from cholesterol, which diffuses across the basement membrane to be used for estrogen synthesis in an FSH-stimulated reaction in granulosa cells (Makris and Ryan, 1975; Fortune and Armstrong, 1977; Tsang and Armstrong 1980;

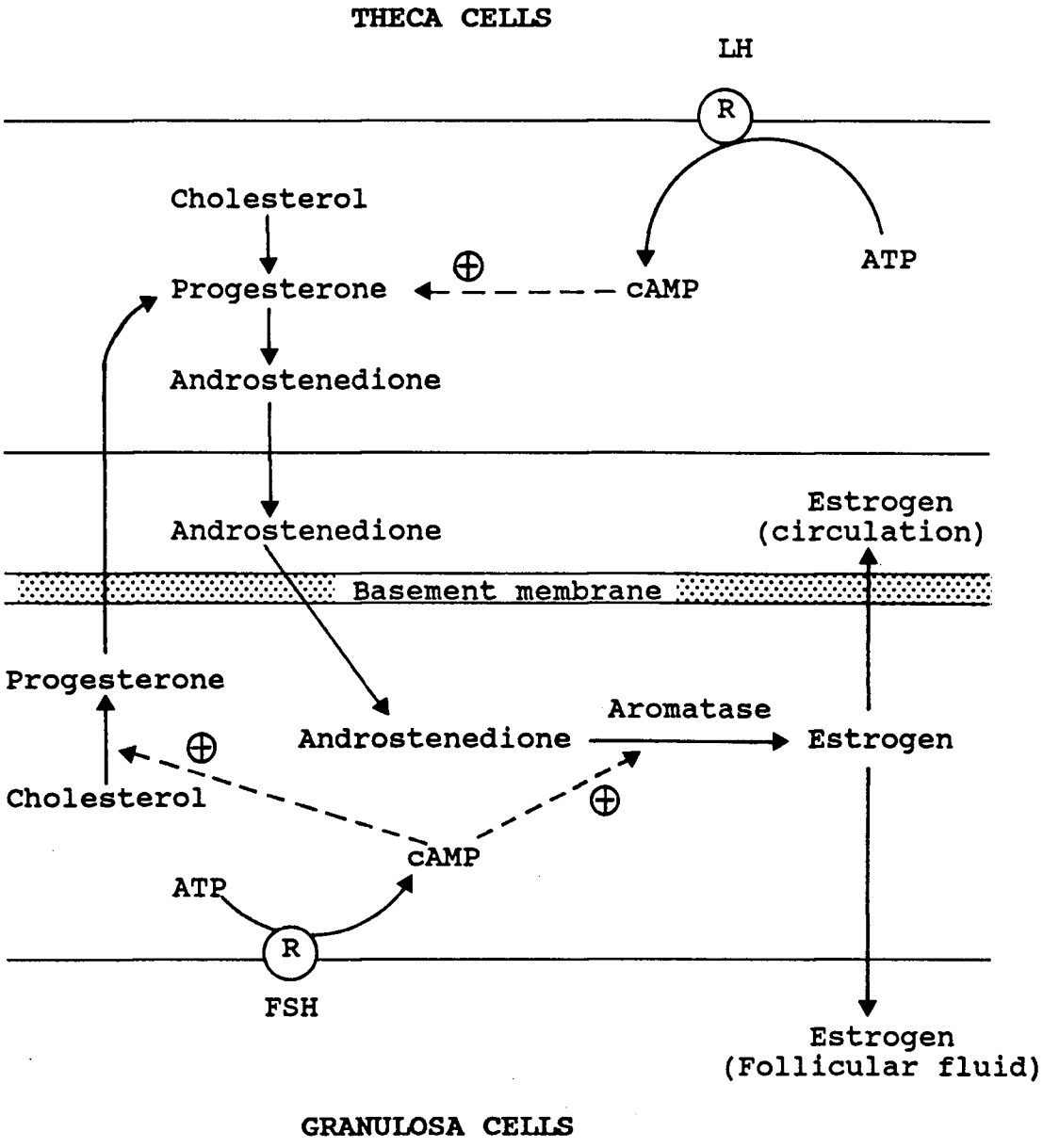

Fig. 3. Diagram of the "two-cell, two gonadotropin theory" of follicle steroidogenesis.

Erickson, 1978). Since granulosa cells secrete P_4 in response to gonadotropins, it is also possible that granulosa cell P_4 may diffuse into theca cells to serve as a substrate for androgen biosynthesis. Theca interna cells convert P_4 to androstenedione by 17-alpha-hydroxylase and $C_{17,\ 20}$-lyase. In contrast, granulosa cells do not have significant activities of C_{21} side-chain cleavage enzymes and synthesize little or no androgens from either P_4 or pregnenolone (Lacroix et al., 1974; Hamberger et al., 1978; Short, 1962; Fowler et al., 1978). On the other hand, granulosa cells do possess considerable 17-beta-HSD activity (Makris and Ryan, 1980; Nimrod et al., 1980; Moon and Duleba 1982), which acts on androstenedione and estrone to form testosterone and estradiol, respectively. Although androstenedione is the major ovarian androgen in most species, the 17-beta-HSD reaction favors the production of estradiol as the major estrogen. These interactions between LH and FSH together with the cyclical changes in plasma concentration of LH and FSH provide a mechanism to account for the regulation of ovarian steroidogenesis and follicular growth.

While the foregoing account has focussed on the roles of LH and FSH in regulating activity of ovarian functions, there is also evidence that a third pituitary gonadotropin, prolactin (PRL), may also regulate ovarian activity at the ovarian level. PRL receptors have been demonstrated in the human ovary (Saito and Saxena 1975), and in rat and porcine granulosa cells and luteal cells (Richards and Williams, 1976; Rolland and Hammond,

1975; Rolland et al., 1976). PRL may interact with granulosa cells to promote their maturation since PRL is essential for maximum production of P_4 by human luteinized granulosa cells in in vitro studies (McNatty et al., 1974). PRL acts as a luteotrophic agent by stimulating P_4 production (Rothchild, 1981; Smith, 1980) as well as by maintaining the level of LH receptor in rat ovary (Holt et al., 1976), and may influence the pool of steroid precursors available for P_4 synthesis (Armstrong et al., 1970; Behrman et al., 1970). Receptors for PRL, like those for LH and FSH, appear to be located on the cell membrane but in contrast to LH and FSH, interaction of PRL with its receptor does not stimulate adenylate cyclase (Mason et al., 1973) and no second messenger for PRL has been convincingly documented.

In addition to steroids, granulosa cells also secrete PGs. PG synthesis is stimulated by LH and FSH resulting in increased production of PGE_2 and PGF_{2alpha} (Clark et al., 1978; Marsh et al., 1974; Knazek et al., 1981; Zor et al., 1983). An ovulatory dose of hCG directly increases the follicular content of PGs (Richards et al., 1982). The rate of production of PGs in granulosa cells is directly proportional to the concentration of hCG used to stimulate the cells (Hedin., et al., 1987). Doses of hCG capable of stimulating ovulation increase PG synthesis, whereas sub-threshold doses of hCG only slightly increase PG synthesis and do not induce ovulation. The increase of PGs induced by hCG is transient in rat ovary (Hedin et al., 1987). The concentrations of PGs reach a

maximum prior to ovulation and return to low levels within 24-48 h following the LH/hCG surge.

B. Intraovarian regulation by follicular steroids

Role of progestins

Granulosa cells synthesize and secrete large quantities of P_4, which may exert some effects on follicular growth and granulosa cell function. In prepubertal rats, exogenous administration of P_4 facilitates the hCG-stimulated growth of small antral follicles and hCG-induced estrogen biosynthesis (Richards and Bogovich, 1982). P_4 also enhances ovarian P_4 secretion by the preovulatory follicle without affecting the level of LH secretion (Uchida et al., 1972). In contrast, in monkeys, unilateral ovarian implants of P_4 directly inhibit follicular growth without affecting the function of the contralateral ovary, suggesting that a locally high concentration of P_4 may inhibit folliculogenesis (Goodman and Hodgen, 1979). Administration of P_4 to hamsters results in a fall in blood estradiol concentration without a change in serum levels of gonadotropins. This decline is not reversed by concomitant administration of testosterone, indicating that P_4 acts at the level of the aromatase (Greenwald, 1974). The P_4 receptors have been identified in the cytoplasm of rat granulosa cells (Schreiber and Erickson, 1979; Naess, 1981). Similar P_4 receptors have been identified in the ovaries of rabbit, cow and human (Philibert et al., 1977; Jacobs and

Smith, 1980; Jacobs et al., 1980). The presence of ovarian P_4 receptors suggests an important intracellular regulatory role for P_4. Other studies have demonstrated a role of P_4 in the autonomy of luteal cell P_4 biosynthesis and in an autocrine control mechanism in which the P_4 production of the cells exerts ultra-short loop feedback regulation of its own production (Goff et al., 1979; Fanjul et al., 1983).

Role of androgens

In addition to serving as substrates for aromatase enzymes to form estrogens, androgens exert a variety of actions in granulosa cells through interaction with intracellular androgen receptors. Pretreatment of intact rats with dihydrotestosterone prevents the FSH induction of LH receptors in granulosa cells and this effect can be antagonized by estrogen treatment (Farookhi, 1980). Although androgen treatment induces atresia in the absence of FSH, androgens augment gonadotropin-stimulated steroidogenesis. Both in vivo and in vitro experiments have shown that androgens stimulate ovarian aromatase activity (Katz and Armstrong, 1976; Daniel and Armstrong, 1980). Androgens also act synergistically with FSH to stimulate progestin production in cultured rat granulosa cells. The stimulatory effect of androgens on progestin biosynthesis appears to be the result of the stimulation of SCC and 3-beta-HSD (Nimrod, 1977; Welsh et al., 1982).

Role of estrogens

Estrogens maintain secondary sexual characteristics and exert feedback action on the hypothalamic-pituitary unit. Moreover, estrogens play a modulating role at the site of its formation. Estrogens have been known to exert a direct anti-atretic effect. The induction of atresia may be associated with a loss of estradiol receptors in granulosa cells (Richards, 1975; Harman et al., 1975; Ingraham, 1959). Estrogens also regulate estrogen production of granulosa cells by augmenting the FSH-induced aromatase activity, and the minimal effective dose ($3.7x10^{-10}M$) of estradiol-17-beta on aromatase activity is within the range of estradiol-17-beta measured in the follicular fluid of rat preovulatory follicles. This suggests that of estrogen plays a physiological role as an end product amplifier of aromatase activity to enhance the synergistic effect of androgens (Goff and Henderson, 1979; Adashi and Hsueh, 1982). Estrogen enhancement of FSH-stimulated granulosa cell aromatase activity may explain the maintenance of dominant follicles in the ovary. On the other hand, estradiol may inhibit production of its precursor androgen through negative feedback on the theca cells (Leung et al., 1978; Leung and Armstrong, 1979). Such an intraovarian negative feedback mechanism may be significant in limiting the estrogen production and provide adequate time for oocyte maturation before ovulation. Local intrafollicular concentrations of estrogen or the ratio of estrogen and androgen may determine which follicle(s) in one cycle will

escape atresia and go on to ovulation (Harmon et al., 1975; Hillier et al., 1980).

C. Role of neurotransmitters on ovarian steroidogenesis

The innervation of the mammalian ovary has been well documented. The dense adrenergic innervation of the mammalian ovary suggests a role for the adrenergic system in the regulation of ovarian functions (Moshin and Pennefather, 1979; Lawrence and Burden, 1980). The possible role of catecholamines in the direct regulation of steroid biosynthesis by follicle cells has been studied both in vivo and in vitro. Catecholamines stimulate P_4 production in cultured luteal and granulosa cells, and the stimulation could be blocked by the $beta_2$-adrenergic antagonist (IPS339), but not practolol ($beta_1$-adrenergic antagonist) or phentolamine (alpha-adrenergic antagonist) (Bahr et al., 1974; Condon and Black, 1976). In vivo studies have shown that beta-adrenergic, but not alpha-adrenergic agonists result in increased P_4 production by the ovary (Bahr et al., 1974). Another neurotransmitter that has been extensively examined in the ovary is gamma-aminobutyric acid (GABA) (Erdo and Lapis, 1982). In whole ovary, GABA concentration is comparable to brain levels and is 5 to 6 fold higher than any other non-neuronal tissues studied. GABA binding sites are elucidated by measuring the specific binding of a GABA agonist, [^{3}H]-muscimol (Schaeffer and Hsueh, 1982). Although the physiological role of GABA in the ovarian tissues remains to be elucidated, production of cAMP in slices of rat

ovary is increased by GABA and this effect is antagonized by GABA receptor blockers (picrotoxin and bicuculline) (Erdo and Lapis, 1982).

D. Regulation of ovarian steroidogenesis and ovarian function by prostaglandins

PGE_2 stimulates cAMP, estrogen and P_4 production, and induces resumption of meiotic division of the oocyte and ovulation. Although PGE_2 can mimic some effects of LH, the action of LH and PGE_2 are independent and parallel. In the presence of cyclooxygenase inhibitors, LH stimulates cAMP accumulation and P_4 production (Linder et al., 1974; Linder et al 1980). Although LH induces the process of ovulation, the final phase of ovulation, follicular rupture, does not occur in the absence of PGE_2. This indicates that the presence of PGs is required for ovulation. Inhibition of PG synthesis by administration of indomethacin blocks ovulation (Armstrong and Grinwich, 1972; Armstrong et al., 1974). The concentration of PGs in the ovaries, follicles and follicular fluid rise as time of ovulation approaches (Linder et al., 1980; Murdoch et al., 1981; Ratwardhan and Lanthier, 1981). The stimulation of plasminogen activator and proteoglycan production in granulosa cells further supports the involvement of PG in the process of ovulation. Plasminogen is a glycoprotein contained in the plasma and is converted to the active serine protease by two different plasminogen activators which can be stimulated by PGF_{2alpha} and PGE_2. Plasmin, which is produced by the action

of plasminogen activator on plasminogen, triggers the various steps in the postulated cascade. The net effect is to decrease the strength of the follicle wall to the point at which rupture occurs (Ossowski et al., 1979; Beers et al., 1975; Espey, 1980). Because the gonadotropins and the prostaglandins stimulate adenylate cyclase, cAMP is probably involved in the activities of protein synthesis, leading to increased production of plasminogen activator (Strickland and Beers, 1976). It has been demonstrated that PGE_2 is involved in reversal of ovum maturation and that PGF_{2alpha} may overcome blockade of ovulation by indomethacin (Downs and Longo, 1982;, 1983). However, it should be noted that PGE_2 affects ovulation in indomethacin-blocked animals (Tsafriri et al., 1972), and thus plays a major role in ovulation. PGE_2 is the predominant PG in the follicles and is responsible for most of the effects of PG on ovulation, but PGF_{2alpha} may exert an effect on the smooth-muscle elements of the follicle wall (Diaz-Infante et al., 1974).

E. Role of local nonsteroidal regulators on ovarian function

Endocrine glands, such as the pituitary, ovary and thyroid glands, release hormones which reach their target via the blood stream and thereby affect other tissues, organs or body functions. Paracrine control mechanisms involve local diffusion of hormones to their neighboring cells without entering the circulatory system (Roth et al., 1983). Finally, the regulatory function of some hormones is autocrine since

effects are exerted on the cells which produce the hormones.

There is increasing evidence to suggest that local nonsteroidal regulators play important roles in the ovary by paracrine or autocrine control mechanisms. These nonsteroidal regulators include LHRH (Hsueh and Jones, 1981), growth factors (Gospodarowicz et al., 1977a; 1977b; 1979), insulin and insulin-like growth factors (Veldhuis et al., 1983; Adashi et al, 1985), ovarian angiogenic factors (Koos and LeMaire, 1983), angiotensin (Culler et al., 1986; Husain et al., 1987), bradykinin (Smith and Perks, 1983), neurotransmitters (Hsueh et al., 1984), oocyte maturation inhibitor (Tsafriri and Braw, 1984) and neurohypophyseal hormones (Sheldrick and Flint, 1984). These local nonsteroidal regulators may interact with gonadotropins, steroid hormone and PGs to regulate steroidogenesis, oocyte maturation and ovulation by paracrine or autocrine mechanisms.

The effects of LHRH on ovarian function have been extensively studied. A direct function of LHRH in the ovary was reported by Rippel and Johnson who observed a decrease in hCG augmented ovarian weight in immature hypophysectomized rats treated with LHRH (Rippel and Johnson 1976). This finding was confirmed in hypophysectomized rats stimulated with pregnant mare's serum gonadotropin (PMSG) or FSH (Ying and Guillemin, 1979; Hsueh and Erickson, 1979). <u>In vitro</u> studies have shown a direct effect of LHRH on primary cultures of granulosa cells as well. Treatment with LHRH or its agonists inhibits FSH-stimulated progestin and estrogen production (Hsueh and

Erickson, 1979; Arimura et al., 1979). The action of LHRH on granulosa cell steroidogenesis is exerted at multiple sites including inhibition of FSH-stimulated cAMP production, inhibition of aromatase, SCC and 3-beta-HSD and stimulation of 20-alpha-HSD. LHRH also suppresses LH and FSH receptors (Hsueh and Jones, 1981; Hsueh et al., 1981; Gore-Langton 1981). The inhibitory action of LHRH on the ovary is exerted on other ovarian compartments in addition to the granulosa cells. LHRH inhibits basal and LH-stimulated androgen synthesis by rat ovarian interstitial cells (Magoffin et al., 1981; Magoffin and Erickson, 1982). Additionally, LHRH inhibits LH/hCG-stimulated P_4 secretion by rat luteal cells in vivo and in vitro (Clayton et al., 1979; Jones and Hsueh, 1980). In contrast to the inhibitory effects of LHRH, stimulatory effects following acute administration of LHRH alone have also been observed. These effects include the stimulation of estrogen, P_4, 20-alpha-OH-P and PGs production (Dorrington et al., 1982; Gore-Langton et al., 1981; Clark et al., 1980; Clark, 1982). Stimulatory and inhibitory effects of LHRH could be blocked by treatment with LHRH antagonists (Jones and Hsueh 1981; Hsueh and Ling, 1979; Navickis et al., 1982). The most consistent stimulatory action of LHRH on ovarian function is exerted on mature preovulatory follicles. LHRH induces ovulation and this action of LHRH is blocked by LHRH antagonists (Ekholm et al., 1982; Dekel et al., 1983). The action of LHRH on follicular rupture at ovulation appears to be related to its ability to stimulate PGs and plasminogen activitor, both of them have been shown previously

to be involved in follicular rupture (Hillensjö et al., 1982 Wang 1983; Reich et al, 1985). Additionally, LHRH is involved in the resumption of ovum maturation and cumulus cell dispersion (Dekel et al., 1981; Hillensjö and LeMaire, 1980; Magnusson and LeMaire, 1981). Unlike its effect on follicular rupture, the action of LHRH on the ovum is not blocked by indomethacin and hence does not seem to be mediated by follicular PG production (Ekholm et al., 1982). Recent studies have proposed that the action of LHRH on ovum maturation involves protein kinase C (PKC). Furthermore, inhibitors of the lipoxygenase pathway of AA inhibits the resumption of meiosis induced by LHRH, but not by LH, indicating the involvement of this pathway in mediating LHRH action on ovum maturation (Tsafriri et al., 1986; Aberdam and Dekel, 1985; Ekholm et al., 1982). The finding of specific receptors for LHRH in the rat oocyte strongly suggests a direct effect of LHRH on oocyte maturation (Dekel et al., 1988).

The direct effcts of LHRH on ovarian steroidogenesis are mediated by its specific receptors. These receptors are found in luteal, theca and granulosa cells at all stages of cellular differentiation (Pelletier et al., 1982). Photoaffinity labeling of ovarian LHRH receptors has identified two specific components with apparent MW of 60,000 and 54,000 daltons (Hazum and Nimrod, 1982; Hazum, 1984). LHRH increases the amount of its own receptor, whereas gonadotropins cause LHRH receptor depletion (Clayton and Catt, 1981). Apart from the hormonal regulation, the ovarian LHRH receptor might also be under a

direct neural control (Marchetti and Cioni, 1988). Since only one type of LHRH receptor is identified in the pituitary, the extra component of the ovarian receptors may be related to the different and specific functions of LHRH-like peptide in the ovary. Although the rat model has been extensively used to study the direct effects of LHRH on gonadal function, other studies have demonstrated direct effects of LHRH on the ovary of rabbit (Koos and LeMaire, 1985), pig (Massicotte et al., 1980), cow (Milvae et al., 1984), chicken (Takats and Hertelendy, 1982), monkey (Knecht et al., 1983), and human (Tureck et al., 1982). The high affinity ovarian LHRH receptors have been demonstrated in rat, but not in sheep, pig, and cow (Brown and Reeves, 1983), monkey (Asch et al., 1981), and human (Clayton and Huhtaniemi, 1982). On the other hand, low affinity LHRH receptors were documented in human corpus luteum (Popkin et al., 1983). The failure to demonstrate high affinity LHRH binding sites in other species might be due to the poor ability of the labeled LHRH analogs used to interact with the ovarian LHRH receptors in these species.

However, the low level of LHRH in systemic blood indicates that LHRH may not be the endogenous ligand that binds to the LHRH receptors in the rat ovary (Aten et al., 1986). To demonstrate the physiological significance of the direct ovarian actions of LHRH, it is necessary to establish the presence of an ovarian LHRH-like substance. Recently, it was shown that rat, bovine and ovine ovaries contain a LHRH-like peptide that competes with LHRH for binding to ovarian membrane

receptors but with immunoreactive activity distinctly different from those of LHRH (Aten et al., 1986; Aten et al., 1987). Interestingly, a separate gonadotropin-releasing peptide has been isolated from human follicular fluid (Li et al., 1987). The amino acid composition and sequence of this latter peptide differ from those of hypothalamic LHRH (with the primary structure of H-Thr-Asp-Thr-Ser-His-His-Asp-Gln-Asp-His-Pro-Thr-Phe-Asn-OH) and this peptide is considerably less potent in stimulating the release of gonadotropins from the mouse pituitary in vitro. The LHRH receptors in the rat ovary may represent receptors for one or more of these LHRH-like peptides found endogenously in the rat ovary. The presence of equivalent levels of LHRH-like peptide in the ovine, bovine and human ovary suggests that LHRH-like peptide might serve a paracrine or autocrine role in these tissues via the receptors specific for LHRH-like peptide. Additionally, a recent report has suggested that porcine inhibin alpha-subunit of 134 amino acid suppresses FSH-induced production of cAMP, P_4 and estradiol via a LHRH receptor in rat granulosa cells, raising further interest in the nature of LHRH receptors in the ovary (Hillier et al., 1987).

IV. Signal transduction systems in ovary

A. Introduction

Ovarian cellular functions are regulated by peptide hormones, neurotransmitters and nonsteroidal factors and these

hormones regulate ovarian cells via second messengers. Generally, the capacity of a given cell to respond to a given hormone depends on the presence or absence of the receptor in the cell. It is well recognized that there are several classes of hormone receptors, which when occupied by their specific hormones, stimulate different second messengers, whose diffusion enables the hormonal signal to spread rapidly throughout the cell. Two major signal pathways are now known. One employs the second-messenger cyclic adenosine monophosphate (cAMP). The other employs a combination of second messengers that includes calcium ions (Ca^{2+}), inositol 1,4,5-trisphosphate (IP_3) and sn-diacylglycerol (DG).

B. Cyclic AMP

A large number of hormones exert their effects by increasing the concentration of cAMP. cAMP is formed from ATP by the membrane bound enzyme adenylate cyclase. Each hormone molecule results in increased formation of many molecules of cAMP. Therefore, the initial hormone signal is greatly amplified following its interaction with plasma membrane-bound receptors. cAMP interacts with a specific intracellular allosteric receptor, the regulatory subunit of cAMP-dependent protein kinase, and upon dissociation of the free catalytic subunit induces the phosphorylation of substrate proteins to give further amplification. The agonist-induced increase in cAMP and subsequent cellular response is terminated by degradation of cAMP to 5'-AMP by the action of

phosphodiesterase, hydrolysis of GTP to GDP by GTPase and removal of phosphate groups from substrate proteins by phosphatase enzymes. It is believed that cAMP is the second messenger for the action of both gonadotropins, LH and FSH, in ovarian cells, and multiple functions of ovarian cells can be elicited by cAMP analogs and cAMP-inducing agents (Kolena and Channing, 1972; Goff and Armstrong, 1979; Marsh and Savard, 1966; Tsang et al., 1979; Dennefors et al., 1980). Since PGE_2 also induces the increase in cAMP levels in cultured granulosa cells, endogenous PGs may also affect granulosa cell differentiation (Kolena and Channing, 1972; Goff and Armstrong, 1977; Behrman, 1979).

Studies performed during the past decade have revealed that the regulation of hormone-sensitive adenylate cyclase is far more complicated than originally suspected. Fig. 4 presents in a scheme many of the structural and functional aspects of adenylate cyclase activity by nucleotides and hormones. Adenylate cyclase is only part of a complex regulatory system that mediates the action of hormones on their target cells. The enzyme system is composed of at least three classes of components. Located at the outer membrane surface is the receptor (R) component containing a specific site for binding of hormones. At the inner face of the membrane are the catalytic unit (C) and the guanine nucleotide regulatory protein (G) (Rodbell, 1980). Receptors communicate with a pair of homologous guanine proteins. One of which (Gs) mediates stimulation of adenylate cyclase activity, while the other (Gi)

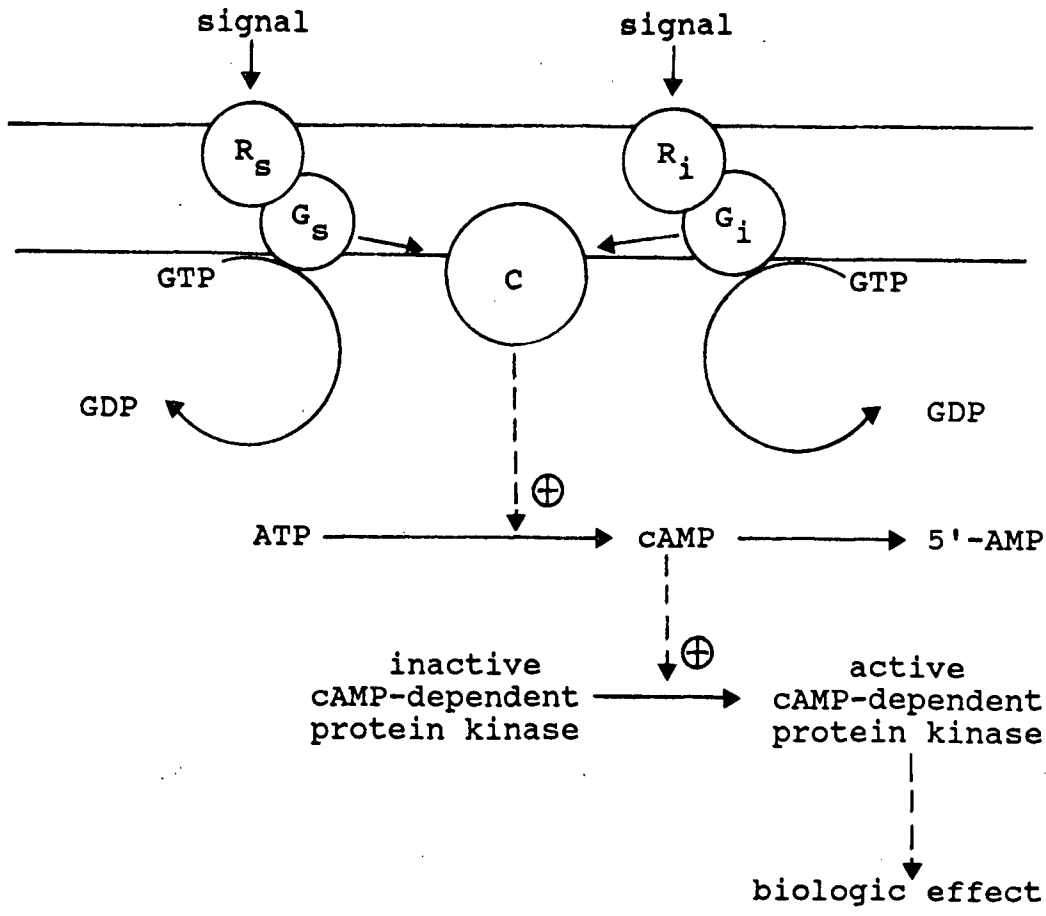

Fig. 4. General model of cAMP mediated hormone response. R, receptor; Gs, stimulatory guanine-binding protein; Gi, inhibitory guanine-binding protein; GTP, guanosine triphosphate; GDP, guanosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; 5'-AMP, adenosine 5'-phosphate.

is responsible for inhibition (Rodbell, 1980; Gilman, 1984). They are both formed of alpha, beta and gamma subunits, both alpha-subunits bind guanosine triphosphate (GTP) and its analogs. When hormone binds to receptor (H•R), there is a rapid interaction of H•R with G to form H•R•G. Formation of H•R•G complex affects G activity, allowing the binding of GTP to its specific binding site to form H•R•G•GTP. G is active

only when GTP is bound; it is inactive when GDP is bound. H•R•G complex increases removal of inhibitory guanosine diphosphate (GDP) and facilitates GTP binding. GTP-dependent activation is represented by concomitant subunit dissociation to give a GTP•alpha complex, which interacts with C to enhance or decrease catalytic activity depending on the type of G protein, and a beta•gamma complex, which does not itself appear to dissociate. Reversal of adenylate cyclase stimulation results from GTP hydrolysis by GTPase, which terminates G-protein activition. GTPase-dependent deactivation is assumed to be completed upon reassociation of alpha subunits with beta•gamma complexes (Rodbell, 1980; Jakobs et al, 1984; Gilman, 1984; 1987).

C. Calcium and protein kinase C pathway

In addition to the cAMP pathway, there is another major signalling pathway that utilizes the membrane phosphoinositides (Fig. 5). So far, the collective term phosphoinositides has been used to describe the three anionic phosphoinositides that contain myo-inositol in their head groups (Berridge, 1981). The most abundant form is phosphatidylinositol (PI) that contains myo-inositol attached to phosphate through the hydroxyl on the 1-position of its inositol head group. The other two members are formed by sequential phosphorylation of hydroxyl groups on the 4- and 5-positions to form phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphates (PIP_2) that is the immediate precursor

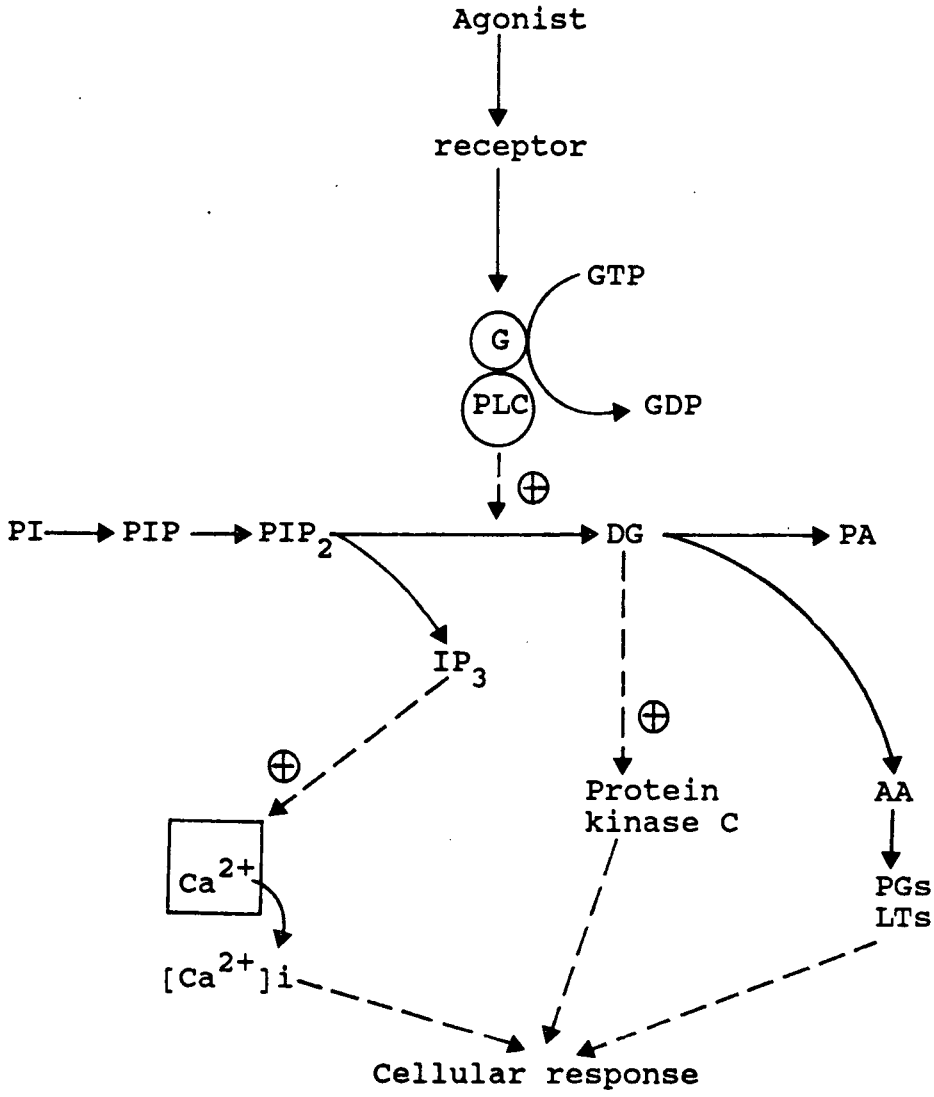

Fig. 5. Inositol phospholipid turnover and signal transduction. Abbreviations: G, guanine nucleotide-binding protein; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; DG, 1,2-diacylglycerol; PA, phosphatidic acid; AA, arachidonic acid; PGs, prostaglandins; LTs, leukotrienes.

located within the plasma membrane used by the receptor mechanism to release inositol 1,4,5-trisphosphate (IP_3) to the cytosol, leaving DG within the plane of the membrane. The inositol phosphates are rapidly degraded to inositol, which is utilized for resynthesis of phosphoinositides, by a complex of

phosphatases, whereas diacylglycerol is converted to either phosphatidic acid or monoacylglycerol plus arachidonic acid (AA). Resynthesis of PI occurs in the membranes of the endoplasmic reticulum where phosphatadic acid interacts with cytidine triphosphate to give cytidine diphosphate-diacylglycerol and this combines with inositol to give PI. PI is then carried back to the plasma membrane by a transfer protein to complete the cycle of breakdown and resynthesis. AA can be derived from membrane phosphoinositides as well as from the sn-2 position of other membrane phospholipids. Since AA availability limits the rate of synthesis of AA metabolites in most tissues, the reactions that produce AA can stimulate lipoxygenase and cyclooxygenase pathways thereby generating other signals, for example, PGs, TXs and LTs. The hydrolysis of inositol lipid is mainly confined to the action of Ca^{2+}-mobilizing agonists, which bind to specific cell-surface receptors and gain access to both intracellular and external sources of Ca^{2+}. Evidence for the IP_3/Ca^{2+}-mobilizing hypothesis has been obtained by studying the effect of this putative second messenger on various permeabilized cells where IP_3 could gain access to the intracellular Ca^{2+} stores, such as endoplasmic reticulum (ER) (Streb et al, 1983; Burgess et al, 1984). Another possible source of Ca^{2+} is from mitochondria. It has been demonstrated that isolated mitochondria participates in the release and uptake of large amounts of intracellular Ca^{2+} (Lehninger, 1970; Carafoli and Crompton, 1978). IP_3 acts through a specific receptor to release Ca^{2+} by

opening a channel across the ER membrane (Smith et al, 1985; Irvine et al, 1984). The initial response to agonists that cause Ca^{2+}-mobilization is a release of internal Ca^{2+} (phase 1), which is soon followed by entry of Ca^{2+} across the plasma membrane (phase II) (Kojima et al, 1985; Reynolds and Dubyak, 1985). Most attention has focused on its role in stimulating the release of Ca^{2+} during cell activation, but IP_3 may serve to regulate the resting or basal level Ca^{2+} as well (Prentki et al, 1985). DG that remains within the plane of the plasma membrane functions as a second messenger by activating protein kinase C (PKC). PKC has been shown to be Ca^{2+}- and phospholipid-dependent for its activity (Nishizuka 1984). One of the important aspects of the activation process appears to be a translocation of PKC from the cytosol into the membrane, and this process might be the role of Ca^{2+} (Wolf et al, 1985). Although the activation of PKC is thought to be biochemically dependent upon Ca^{2+}, it can be physiologically activated independence of Ca^{2+} under some conditions. It is now clear that there is more than one species of PKC molecule, and seven subspecies of PKC have been identified (Nishizuka, 1988). The various subspecies of PKC have different enzymatic properties. The gamma and alpha-subspecies of PKC are much less activated by DG in the presence of phosphatidylserine than is the mixture of beta-1 and beta-2 subspecies, which shows substantial activity in the absence of Ca^{2+} (Nishizuka, 1988). It has also been proposed that different subspecies of PKC are also activated by the series of phospholipid metabolites, such as

DG, AA and lipoxin A (Hansson et al, 1986, Nishizuka, 1988). Once PKC has been activated through the concerted action of DG and Ca^{2+}, it begins to phosphorylate specific proteins that are thought to contribute to the control or modulation of many metabolic and other processes (Nishizuka, 1986).

IP_3 and DG are released from membrane phosphoinositides by a phosphoinositide-specific phospholipase C (PLC). There is convincing experimental evidence at present which suggests a role for GTP-binding protein serving to couple receptors to PLC. An example of the evidence indicating a role for G-protein in the coupling various receptors to PIP_2 hydrolysis and Ca^{2+} mobilization is the finding that nonhydrolyzable analogues of GTP stimulate breakdown of PIP_2 and PLC activity (MaJerus et al, 1986). The identity of this G-protein and its relationship to other G-proteins is unknown.

LHRH is a peptide hormone and its effects are mediated by specific receptors. The mechanism of LHRH action on the ovary has been investigated in the past few years. There is no convincing evidence suggesting that cAMP is the second messenger for LHRH action in the ovary. On the other hand, LHRH and its agonists have been shown to stimulate the breakdown of polyphosphoinositides into inositol phosphates and DG in the ovary (Leung et al, 1983; Naor and Yavin, 1982; Ma and Leung, 1985; Minegishi and Leung, 1985). Ca^{2+} is required in the action of LHRH in the granulosa cells (Ranta et al, 1983) and protein kinase C has been characterized in the ovary (Noland and Dimino, 1986; Davis and Clark, 1983). Recently,

the effect of LHRH on [^{3}H]AA release in rat ovarian cell has also been examined (Minegishi and Leung, 1985). Thus, at the level of the ovarian cell, the hydrolysis of inositol lipids may immediately follow LHRH receptor occupancy and lead to the rapid generation of IP_3 and DG, and the release of AA. The resultant changes in Ca^{2+} mobilization and/or PKC activity and AA metabolism may well be correlated with the modulatory effects of LHRH on ovarian steroidogenesis.

V. The aim of the present study

Although many reports have indicated that LHRH or LHRH-like substance directly affect rat ovarian function, the mechanism of action of LHRH is not completely understood. Since LHRH has been shown to induce membrane phosphoinositide breakdown, the overall aim of the present study is to further test the effects of LHRH on hormone production in rat granulosa cells and investigate the possible signal transduction roles of PKC, Ca^{2+}, AA and its metabolites in the action of LHRH. Specifically, the action of LHRH was compared with that of gonadotropins and cAMP-stimulating agents on the membrane phosphoinositide turnover. Other experiments was examined LHRH-induced $[Ca^{2+}]i$ alteration in individual granulosa cells, as well as the interactions among the putative signal transduction pathways on the regulation of P_4 and PGE_2 production. The objective of the present study was therefore to understand, more completely, the role of LHRH as a paracrine or autocrine regulator of ovarian functions.

Chapter 2. Induction of Polyphosphoinositide Turnover and Arachidonic Acid Release by LHRH

I. Introduction

Numerous studies have shown that LHRH and its synthetic agonists could directly affect steroid hormone production in the ovary (Hsueh and Jones, 1981; Leung, 1985). The direct effects of LHRH on the ovary are mediated by specific receptors (Pelletier et al., 1982). These extrapituitary intraovarian actions are either stimulatory or inhibitory, depending on the duration of LHRH treatment as well as the simultaneous presence of other ovarian cell regulators (such as gonadotropins) during the culture period. While the influence of LHRH on ovarian hormone production is well documented, its mechanism of action at the postreceptor level is still largely unresolved. In the past few years, LHRH and its agonists have been shown to stimulate the breakdown of polyphosphoinositides into inositol phosphates and DG in the ovary (Leung et al., 1983; Naor and Yavin, 1982; Davis et al., 1986; Ma and Leung, 1985; Minegishi and Leung, 1985; Leung et al., 1986). Inositol phosphates, especially IP_3 are known to induce mobilization of calcium ions from intracellular stores (Burgess et al., 1984). On the other hand, DG is now widely accepted to be a potent activator of PKC (Nishizuka et al., 1984). Calcium ion (Ca^{2+}) is required in the action of LHRH in ovarian cells (Ranta et al., 1983; Dorflinger et al., 1984), and PKC has recently been characterized in the ovary (Noland and Dimino, 1986; Davis and

Clark, 1983). Recently, a LHRH-like peptide has been demonstrated in rat, bovine, ovine and human ovaries, further strengthening the concept that LHRH or LHRH-like peptide plays a role in mediating ovarian functions. Thus, within the ovarian cells, the hydrolysis of inositol lipids may immediately follow LHRH receptor occupancy and lead to the rapid generation of IP_3 and DG. The resultant changes in calcium mobilization and/or the activity of PKC may be correlated with the modulatory effects of LHRH on ovarian hormone production. A similar mechanism involving inositol lipid breakdown has been proposed for LHRH stimulation of gonadotropin release in the anterior pituitary gland (Raymond et al., 1984; Huckle and Conn, 1987; Harris et al., 1985; Conn et al., 1985).

In the present study, the actions of LHRH on inositol phosphates, diacylglycerol and arachidonic acid formation were further investigated. Specifically, the action of LHRH was compared with that of gonadotropins and cAMP-stimulating agents on the membrane phosphoinositide turnover. The role of PKC activation in regulating production of inositol phosphates, DG and AA was emphasized in this study.

II. Materials and Methods

Animals

Immature Sprague-Dawley female rats purchased from Charles River Canada, Inc. (Montreal, Canada,) or Animal Care (University of British Columbia) were injected subcutaneously on the 23th day after birth with 12 IU pregnant mare's serum gonadotropins (PMSG) between 09:00 and 10:00 in the morning to stimulate the formation of multiple preantral follicles and provide large numbers of relatively homogenenous granulosa cells at the same stage of development. The rats were killed by cervical dislocation after 48h and the ovaries were removed by surgery.

Preparation of granulosa cells

Granulosa cells were harvested under the dissecting microscope, by puncturing the ovarian follicles with a 27G½ gauge hypodermic needle as previously described (Leung and Armstrong, 1978). The ovaries were squeezed gently and the granulosa cells released into Minimum Essential Medium with Eagle's salts and supplemented with 2 mM of L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin sulfate, and 5 ml of nonessential amino acids (MEM; Gibco, Grand Island, NY). After removal from the ovaries, the cells were expressed through a fine sterilized mesh. The cells were recovered by centrifugation (5 min at 200xg), washed once, and suspended in MEM.

Radiolabeled diacylglycerol and arachidonic acid liberation

In some experiments, granulosa cells ($5x10^5$ cells/ml) were added to 24 well culture plates (Falcon) and were labeled by incubation for 24h in medium containing with 0.2 µCi/ml or 0.5 µCi/ml [5,6,8,9,11,12,14,14,15,-^{3}H]Arachidonic acid (60 Ci/mmol; New England Nuclear, Boston, MA) in MEM containing 5% fetal bovine serum (FBS). The cells were then washed thoroughly and incubated for a further 30 to 60 min in MEM without FBS. At this time, different hormones were added. The various preparations were incubated for different time intervals. At the end of the incubation, the medium was removed and the cells were scraped directly into 1 ml of ice-cold methanol. The lipids in the cells were extracted by the method of Folch et al. (1957). Briefly, 1 ml of methanol was mixed with 2 ml of chloroform and 0.6 ml of water, and mixed on a vortex vigorously. The lower chloroform phase was removed and 1 ml of chloroform was added for the second extraction. The pooled chloroform layer of the two extractions was evaporated to dryness under nitrogen and the residue redissolved in chloroform and methanol (2:1) for thin layer chromatography (TLC). The fatty acids in the culture medium were extracted by the method of Borgeat and Samuelsson (1979). After addition of 1.5 ml of methanol to the medium, the bulk of the precipitated material was centrifuged. The supernatant was collected, and the pellets were washed once with 0.5 ml of methanol. The pooled methanol supernatants were acidified to pH 3 and mixed with 6 ml of diethyl ether. Then 4 ml of

distilled water were added and mixed. After separation of the phases, the water-methanol mixture was removed. The ether phase was evaporated to dryness under nitrogen and the residue dissolved in a 2:1 mixture of chloroform-methanol. The [^{3}H]-labeled AA was isolated by TLC on silica gel 60F-254 plates (Merck, Rahway, NJ) with solvent containing iso-octane-ethyl acetate-water-acetic acid (5:11:10:2) vol/vol, as described previously by Minegishi and Leung (1985). The R_f value of AA was 0.85 with pure standards as reference. Radiolabeled diacylglycerol was separated by TLC with a solvent system containing benzene-diethylether-ethanol-ammonia-water (50:40:2:0.1) vol/vol, as described by Kaibuchi et al. (1983). The areas of the plate corresponding to DG (R_f =0.72) were cut out and their radioactivity determined by liquid scintillation spectrometry.

Analysis of inositol phosphates

Granulosa cells ($5x10^5$ cells/ml) were prelabeled by incubation for 24h in MEM containing myo-[2-^{3}H]inositol (5 µCi/ml) (New England Nuclear; 16.5 Ci/mmol) and 5% FBS for 24h. The cells were then washed and incubated for an initial 10 min in radiotracer-free MEM. At this time, hormones were added (in a 10 µl volume), and the cells were incubated for different times. Lithium chloride (Li^+; 10 mM), which inhibits inositol-1-phosphatase, was added to the medium prior to hormonal treatment, enhancing inositol phosphate accumulation. Incubation was terminated by scraping cells directly into 1 ml

of ice-cold methanol. For extraction, another 2 ml of chloroform and 5 µl of concentrated HCl were added. The final ratio of chloroform/methanol/water was 2:1:0.6. After vortex and removal of the top layer (aqueous), another 0.6 ml of water was added and the extraction was repeated. The two extractions were combined, and the radiolabeled inositol phosphates in the aqueous phase were analyzed by anion exchange chromatography using disposable columns containing 0.5 ml of Dowex AG1-X8 resin (BioRad, 200-400 mesh, formate form). The resin was washed with 0.1 M formic acid/5 mM inositol before use. Aliquots (2ml) of the cell lysates were loaded at 4°C. Free inositol was washed out with water (10 bed volume of resin) whereas sequential washes with 0.1 M formic acid containing 0.2, 0.4, and 1.0 M ammonium formate progressively eluted IP, IP_2 and IP_3, respectively, as described by Downes and Michell (1981). Fractions (2.5 ml) were collected and radioactivity of each fraction was counted following the addition of 15 ml of scintillation fluid (Fisher Scientific, USA).

Hormone and drug preparation

Granulosa cells were treated with various hormones and drugs. LHRH and CT were dissolved in saline. AA, 4-alpha-12,13-didecanoate and 12-O-tetradecanoylphorbol-13-acetate (TPA) were dissolved in ethanol. All drugs were diluted to their respective working concentrations with MEM before use and added in 5 µl aliquots to a total incubation volume of 1 ml. Control incubations received the same volume of ethanol. The

final concentration of ethanol in the incubations did not exceed 0.5%, and ethanol did not influence membrane phospholipid metabolism.

Reagents

The following were purchased from Sigma: lithium chloride, myo-inositol, formic acid, phospholipase C, AA, TPA, 4-alpha-phorbol 12, 13-didecanoate, LHRH and CT. Ammonium formate was from Fisher Scientific Inc. Ovine LH (NIDDK oLH-25), LHRH and PMSG were gifts from the National Hormone and Pituitary Program NIDDKD, NIH. Iso-octane, ethyl acetate, benzene, diethylether, methanol and chloroform were purchased from BDH Inc. (Canada). Acetic acid and ammonia water were purchased from Canlab (Travenol Canada Inc.).

Statistical analysis

Statistical significance of the data was determined by Student's T-test or analysis of variance followed by Scheffe's multiple range test. In all cases, identical or similar results were observed in at least three or more independent experiments. All results were presented as the mean ± SE of determinations from triplicate cultures of cells within each treatment group. A P value of less than 0.05 was considered significant.

III. Results

Effects of LHRH on inositol lipid breakdown and arachidonic acid release

In granulosa cells prelabeled with [^{3}H]-AA, treatment with LHRH caused a significant increase ($P<0.01$), in the levels of radiolabeled DG and AA in the cellular extracts. As illustrated in Fig. 6, addition of LHRH (10^{-6}M) for 5 min stimulated the liberation of [^{3}H]-DG and unesterified [^{3}H]-AA from prelabeled phospholipids, by about 4 and 2.7 fold, respectively, compared with control incubations. Furthermore, in cells prelabeled with [^{3}H]-inositol, treatment with LHRH for 5 min caused a significant increase in accumulation of inositol phosphates ($P<0.01$).

Effect of LHRH on [^{3}H]-labeled diacylglycerol formation

The effect of LHRH on [^{3}H]-DG formation was further examined in cultured granulosa cells prelabeled with [^{3}H]-AA and later exposed to LHRH. As shown in Fig. 7, LHRH enhanced the intracellular DG formation by 1.79 fold. The basal level of [^{3}H]-DG in the medium was much lower than that in the cells and [^{3}H]-DG level did not increase in the medium after the treatment of granulosa cells with LHRH for 3 min.

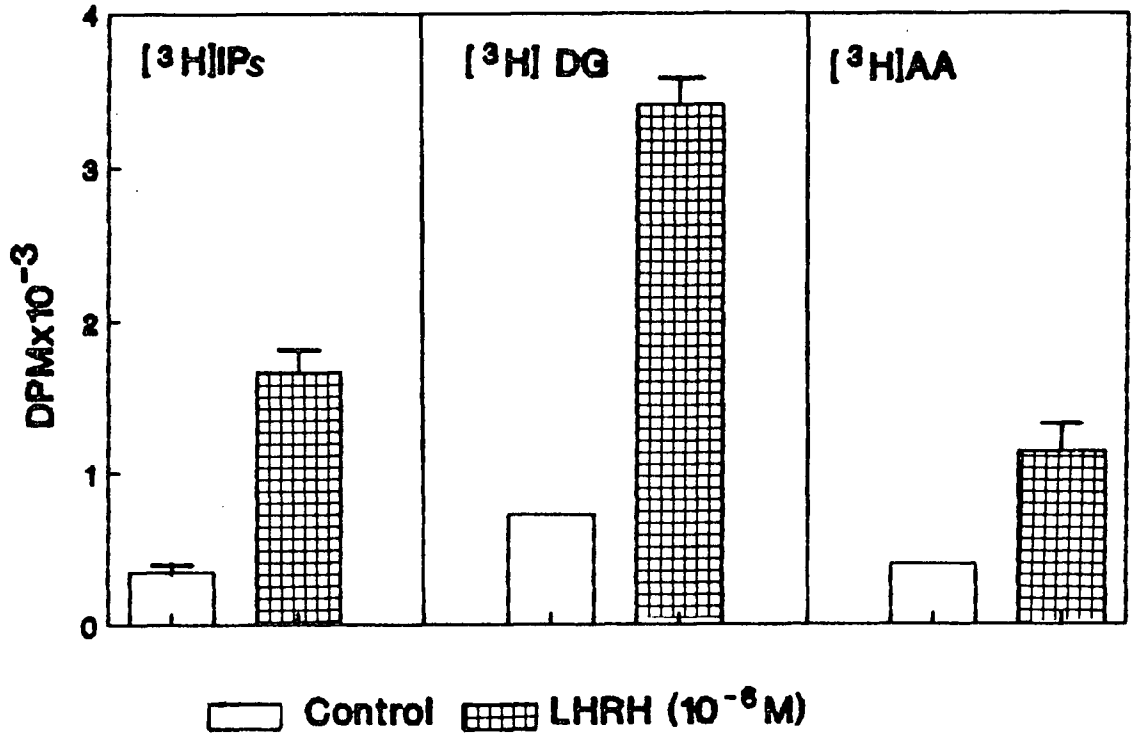

Fig. 6. Stimulatory effects of LHRH on the formation of inositol phosphates (IP_s), diacylglycerol (DG), and the release of unesterified arachidonic acid (AA) in rat granulosa cells. The cells were prelabeled with either [^{3}H]-inositol or [^{3}H]-arachidonic acid, as described in Materials and Methods, and treated with LHRH for 5 min.

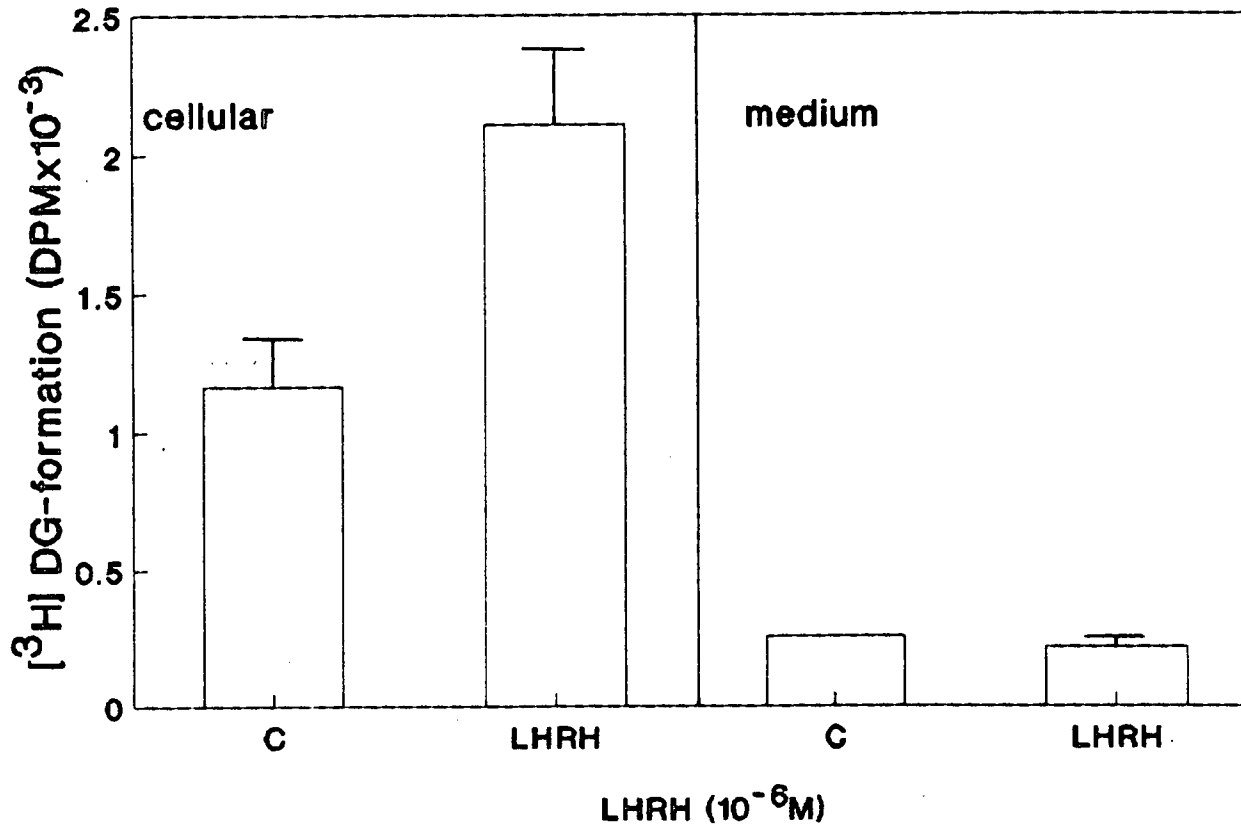

Fig. 7. Effect of LHRH on [^{3}H]-diacylglycerol (DG) formation. The cells were treated with LHRH for 3 min and diacylglycerol (DG) formation was detected from the cells and the medium. In this and subsequent figures, the absence of standard error bars in some of the data points indicates values too small to be shown.

Time response of [^{3}H]-labeled diacylglycerol to LHRH

Fig. 8 shows the time course of [^{3}H]-DG formation in granulosa cells in response to LHRH. LHRH (10^{-6}M) caused a significant increase in [^{3}H]-DG formation, which could be observed as early as 15 sec after LHRH addition ($P<0.05$). In the LHRH treated cells, DG levels continued to increase to about 197% above the control level at 5 min. This declined to about 40% of the 5 min level at 10 min. However, the level of [^{3}H]-DG was still considerably higher (190%) than the control level ($P<0.01$) at 10 min after the treatment. The control levels of DG did not change during the 10 min experiment period.

Effects of LH and LHRH on [^{3}H]-labeled inositol phosphates and diacylglycerol formation and arachidonic acid release

Fig. 9 illustrates that the presence of 10^{-6}M LHRH for 3 minutes markedly stimulated the accumulation of radiolabeled IP, IP_2, and IP_3 to 155%, 545% and 100%, respectively, when compared with untreated control levels. In contrast, LH (1 µg) did not stimulate the formation of inositol phosphate from [^{3}H]-inositol prelabeled granulosa cells in the same experiment. A similar result was also observed for the formation of [^{3}H]-DG (Fig. 10, panel A). In addition, LHRH (10^{-6}M) significantly stimulated AA release, whereas LH (1 µg) did not affect AA release (Fig. 10, panel B).

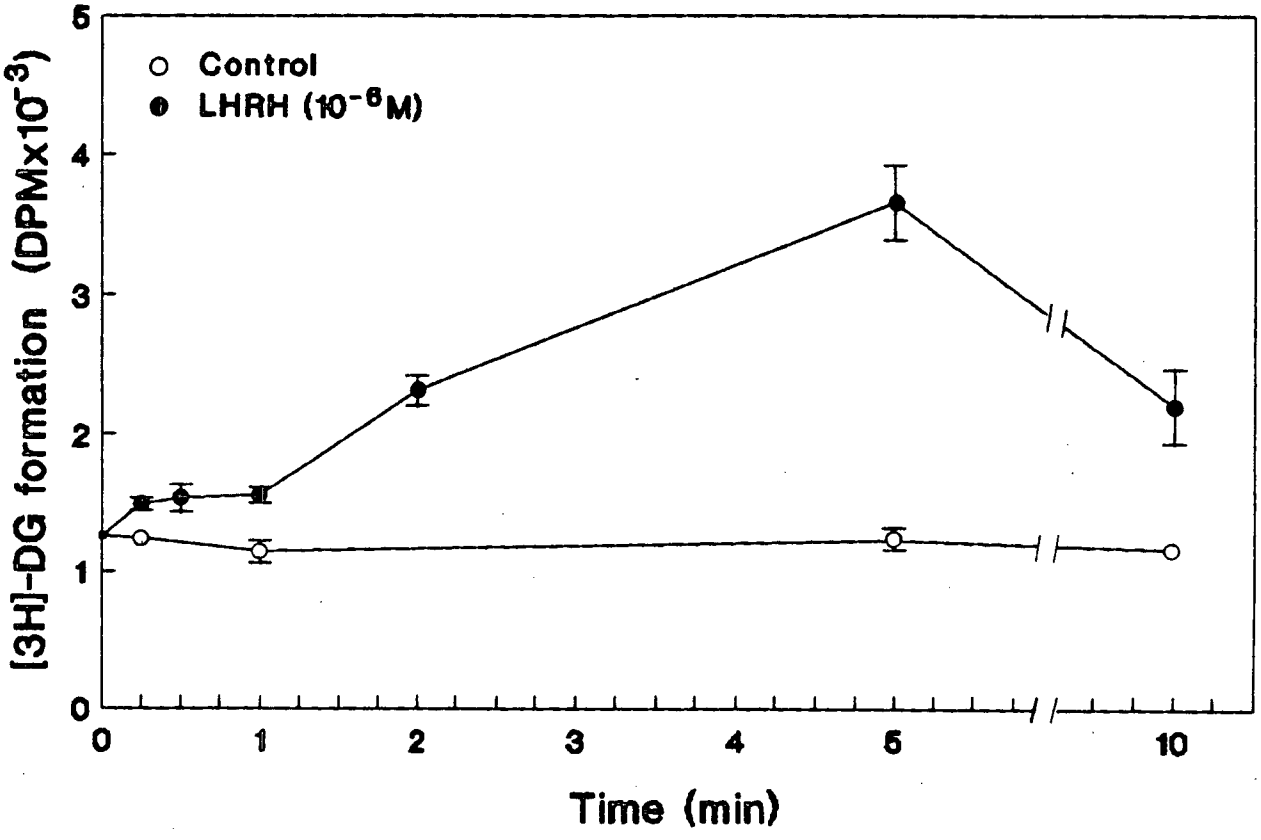

Fig. 8. Time response of stimulation of [^{3}H]-diacylglycerol (DG) formation by LHRH. A significant increase in DG formation was observed as early as 15 second after LHRH addition.

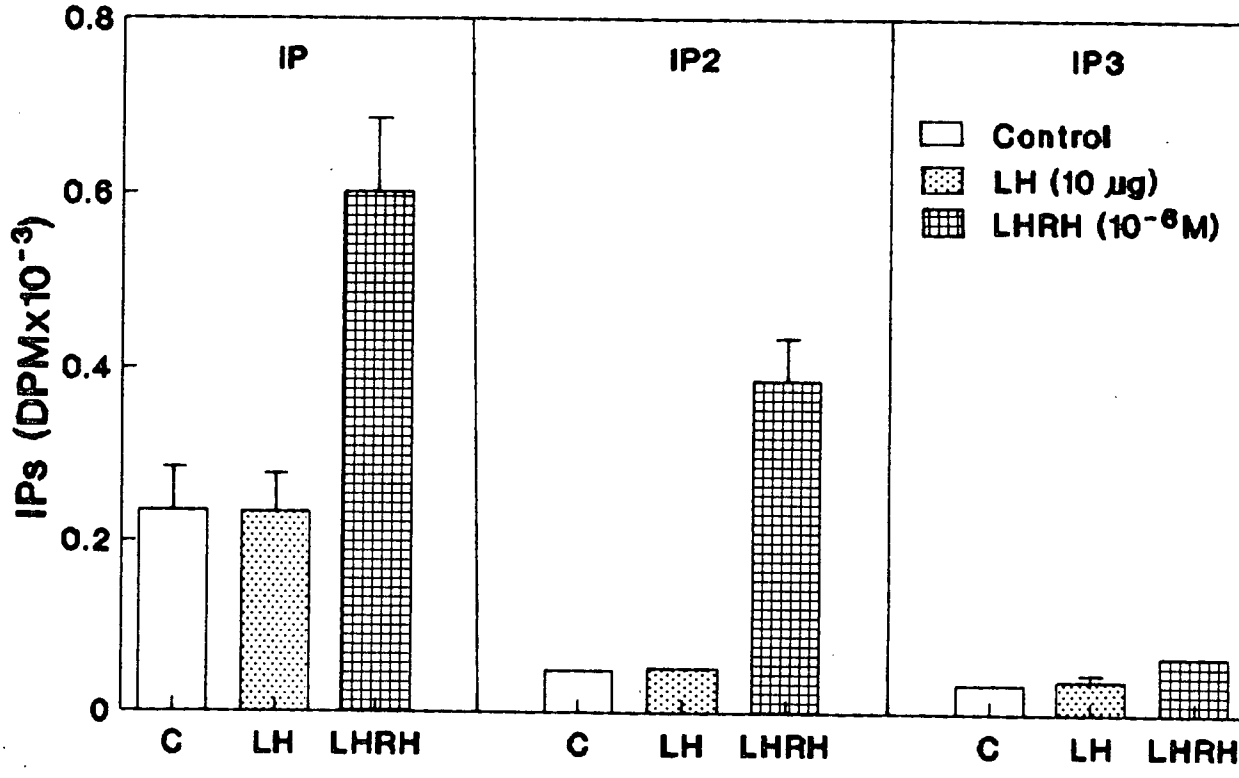

Fig. 9. Comparison of LH and LHRH on [^{3}H]-labeled inositol phosphates. The cells were treated with LH and LHRH for 3 min. LHRH markedly stimulated the formation of inositol phosphates, whereas LH was ineffective.

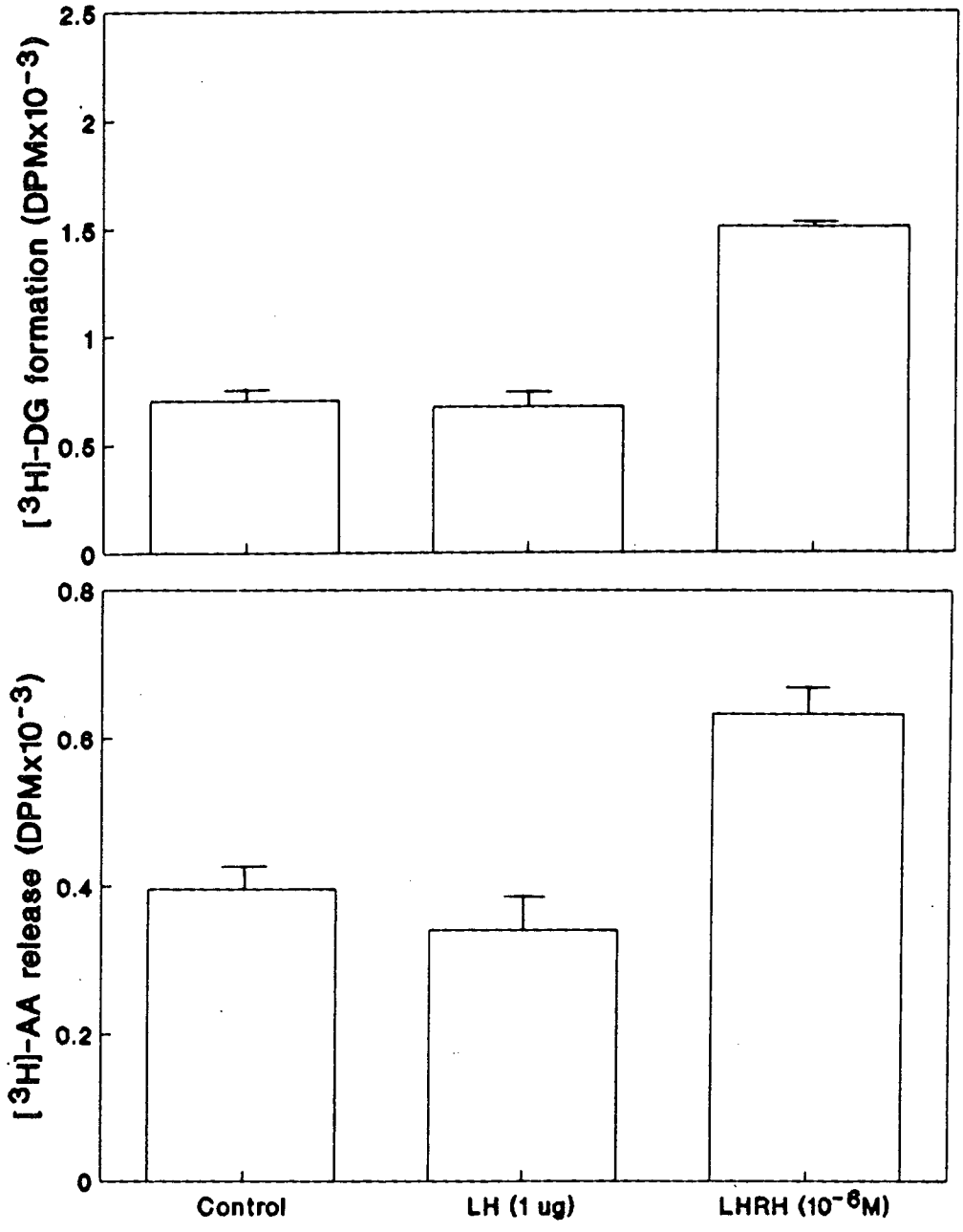

Fig. 10. Comparison of LH and LHRH on diacylglycerol (DG) formation and arachidonic acid (AA) release. The cells were treated with LH and LHRH for 3 min. The addition of LHRH caused significant diacylglycerol (DG) formation and AA release, whereas LH did not alter the formation of these compounds.

Effects of cholera toxin and LHRH on [^{3}H]-labeled inositol phosphates formation

As shown in Fig. 11, in prelabeled granulosa cells with [^{3}H]inositol, the addition of LHRH produced increases in cellular IP, IP_2 and IP_3 (about 380%, 660% and 191%, respectively), during a 5 min period, while addition of CT (100 ng) failed to affect the formation of inositol phosphates.

Effect of phospholipase C on [^{3}H]-diacylglycerol formation

The effect of exogenous phospholipase C on [^{3}H]-labeled DG accumulation was investigated in a separate experiment. Like LHRH, addition of 100 mU PLC resulted in a marked increase in intracellular DG (5.6 fold as compared to control level), whereas a maximal dose of LHRH (10^{-6}M) caused a 1.8 fold increase in the formation of DG (Fig. 12).

Effects of phorbol ester TPA on inositol phosphate and DG formation

To determine whether the activation of PKC by LHRH exerts a possible feedback effect on the hydrolysis of membrane phosphatidylinositides, the granulosa cells were pretreated with a specific PKC activator, TPA, for 5 min and then challenged with 10^{-6}M LHRH for a further 3 min. Significant increases of IP, IP_2 and IP_3 formation were observed when the cells were stimulated with TPA. As demonstrated in Fig. 13, LHRH (10^{-6}M) stimulated formation of IP, IP_2 and IP_3 and this response was unaffected by pretreatment of cells with TPA.

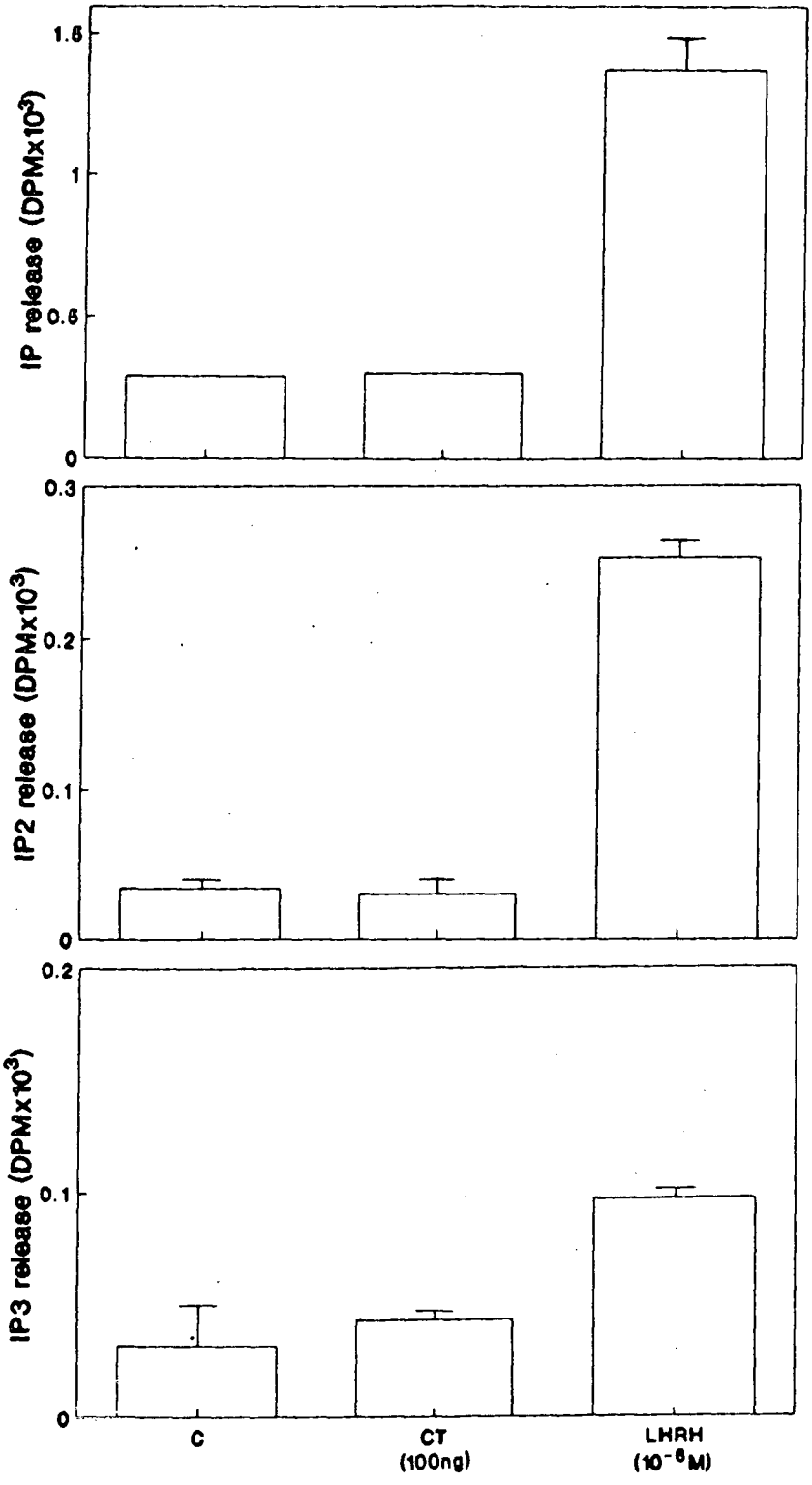

Fig. 11. Effect of cholera toxin (CT) and LHRH on [^{3}H]-labeled inositol phosphate formation. The cells were treated with CT and LHRH for 5 min. The formation of inositol phosphates was stimulated by LHRH but not CT.

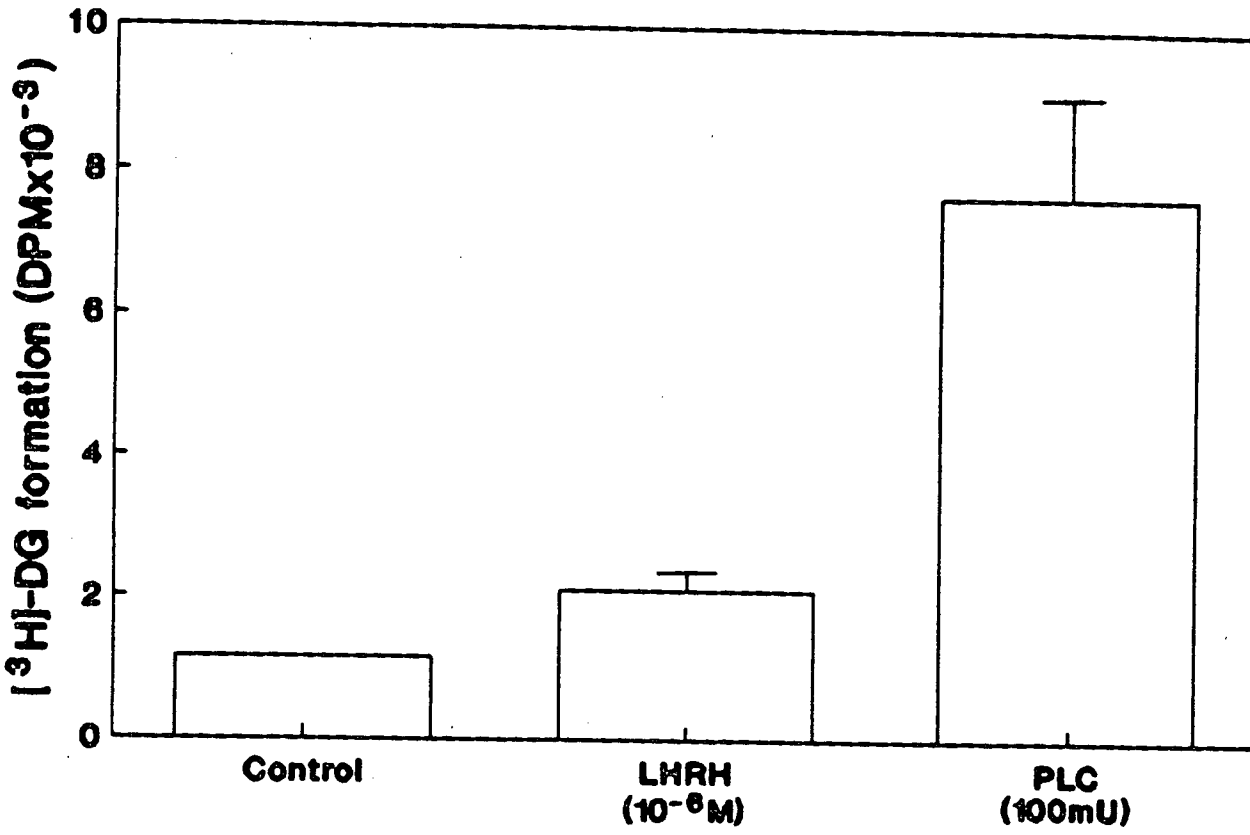

Fig. 12. Effect of phospholipase C (PLC) on [^{3}H]-diacylglycerol (DG) formation. The cells were treated with PLC and LHRH for 3 min. Both PLC and LHRH significantly increased the formation of DG.

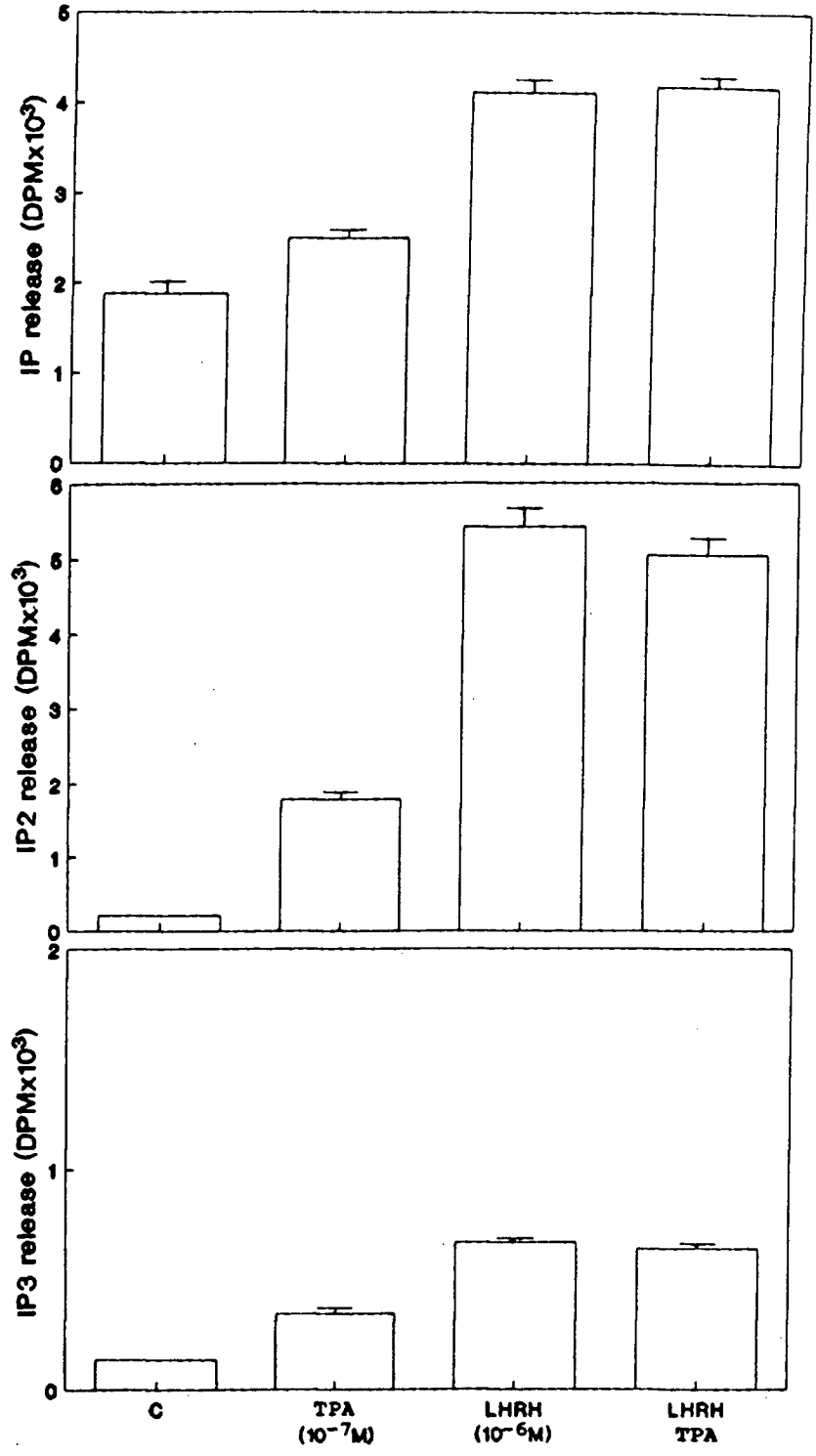

Fig. 13. Action of the phorbol ester TPA on inositol phosphate formation. The cells were first treated with TPA for 5 min, and then treated with LHRH for further 3 min. TPA alone stimulated inositol phosphate formation, but the pretreatment of TPA did not alter the response of the cells to LHRH.

The effect of TPA on DG formation was determined in another experiment in which granulosa cells were prelabeled with [^{3}H]-AA. TPA increased the formation of DG and this action of TPA was specific, since another phobol congener, 4-alpha-phorbol 12, 13-didecanoate, did not stimulate the formation of DG (Fig. 14).

Interaction of the calcium ionophor A23187 and the phorbol ester TPA on arachidonic acid release

To determine the possible interaction between the calcium ionophore A23187 and the phorbol ester TPA on AA release, [^{3}H]-AA prelabeled granulosa cells were treated with TPA and A23187, following a 5 min incubation, A23187 at 10^{-7}M caused a significant stimulation of [^{3}H]-AA release (80% of control, $P<0.05$). However, TPA used alone in similar concentration showed no such effect on AA release. Interestingly, when both A23187 and TPA were present, the effect of A23187 was potentiated ($P<0.05$), with the level of [^{3}H]AA release reaching 130% of control levels (Fig. 15).

IV. Discussion

The present results (Fig. 6) further strengthen the previous findings that LHRH causes a rapid breakdown of inositol lipids in rat ovarian cells. This mechanism was first proposed when LHRH was shown to cause a rapid and selective incorporation of ^{32}P into phosphatidylinositol and phosphatidic acid in rat granulosa cells (Naor and Yavin 1982; Minegishi and

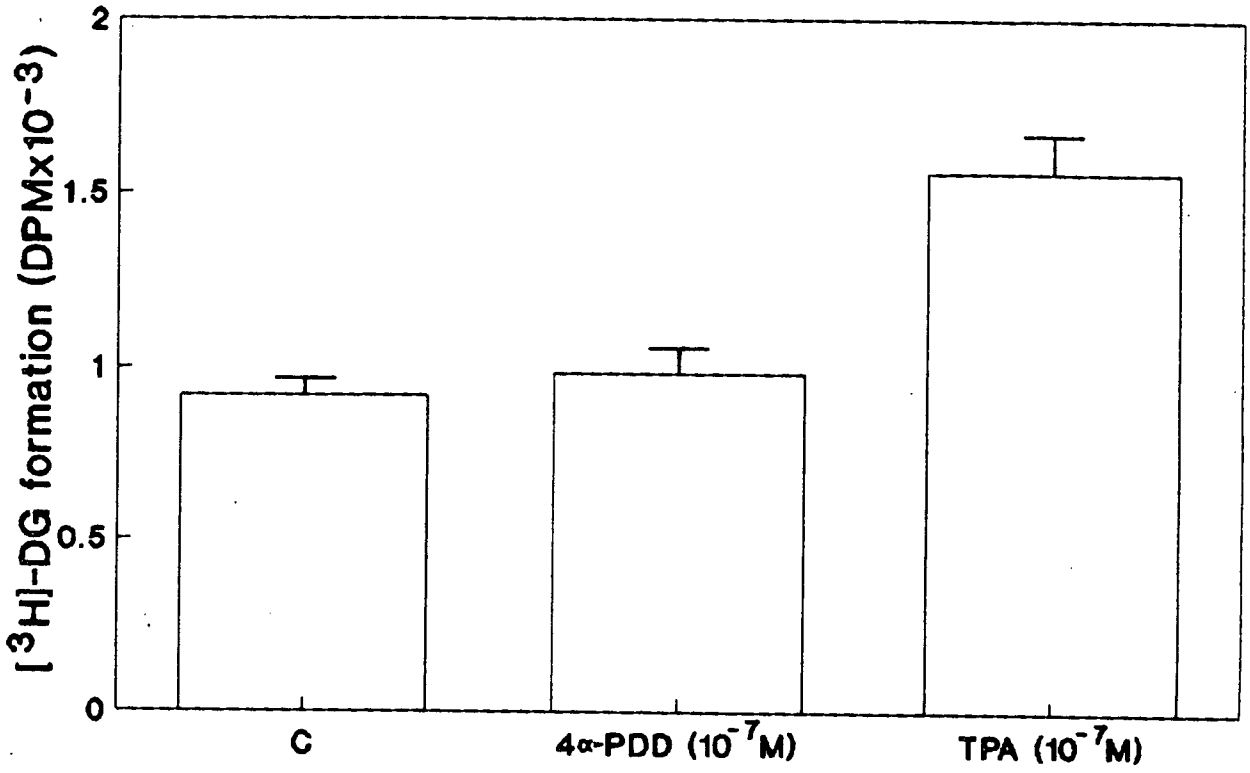

Fig. 14. Specificity of the phorbol ester TPA action on diacylglycerol (DG) formation. The action of TPA on DG formation was specific, since another phorbol congener, 4alpha-phorbol 12, 13-didecanoate (4alpha-PDD), did not change the formation of DG.

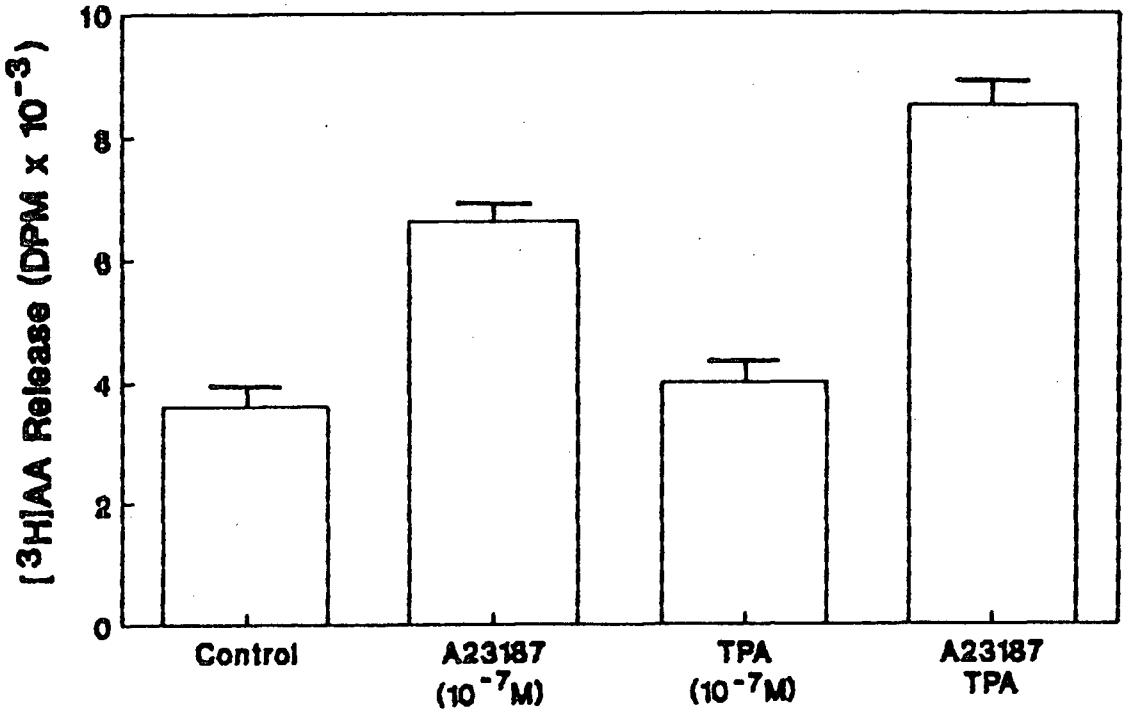

Fig. 15. Interaction of the calcium ionophore A23187 and the phorbol ester TPA on arachidonic acid (AA) release. TPA alone did not alter the release of AA, but potentiated the action of A23187.

Leung, 1985; Leung et al., 1983). Subsequently, it has been demonstrated that the accumulation of the inositol lipid breakdown products, IP, IP_2 and IP_3 is markedly increased following the addition of LHRH to granulosa cells (Ma and Leung, 1985; Davis et al., 1986). The inositol phosphates produced in response to LHRH were from polyphosphoinositol hydrolysis, since LHRH caused a decrease in the level of radiolabeled polyphosphoinositides, while increasing ^{32}P labeling to phosphatidylinositol and phosphatidic acid (Leung et al., 1986). The action of LHRH on ovarian inositol phosphate formation is similar to the action of LHRH on pituitary gonadotropes. In pituitary cell cultures prelabeled with [^{3}H]inositol for 5h, addition of LHRH resulted in an increase in the rate of IP_3 turnover (Huckle and Conn, 1987). Thus, products of polyphosphoinositide breakdown may serve as primary mediators of the early intracellular signal transduction for LHRH both in ovarian and pituitary cells. The formation of IP_3 may be responsible for some of LHRH-induced cellular biological responses. In fact, IP_3 has been shown to induce Ca^{2+} mobilization from intracellular pools (Nishizuka, 1984; Burgess et al., 1984). There apparently is no direct role for IP_3 in regulating calcium entry across the plasma membrane. However, preliminary studies have provided indirect evidence that inositol 1,3,4,5-tetrakisphosphate (IP_4) has a role in the stimulation of Ca^{2+} influx (Berridge, 1987). IP_4 is formed from IP_3 by a IP_3-kinase that transfers a phosphate from ATP to the 3-position of IP_3. In the ovary, both the

inhibitory and the stimulatory actions of LHRH on P_4 production have been shown to be Ca^{2+} dependent (Erickson et al., 1986; Leung and Wang, in press).

Since phosphodiesterase cleavage of PIP_2 is the only known mechanism for IP_3 formation in mammalian cells, LHRH induced PIP_2 breakdown must occur through the action of a polyphosphoinositide-specific phospholipase C (PLC). It has been proposed that thyrotropin-releasing hormone action on phosphatidylinositide breakdown in cultured GH cells occurs via PLC activation (Conn et al., 1987). In rat luteal cells, the addition of exogenous PLC mimicked the action of LHRH and PGF_{2alpha} on the formation of inositol phosphates (Leung et al., 1986). Similar results were also observed in the present study (Fig. 12).

The action of LHRH on inositol phosphate formation in granulosa cells is specific. The earlier studies have shown that LHRH-induced formation of inositol phosphate can be completely blocked by LHRH antagonists, suggesting a receptor mediated mechanism. Furthermore, gonadotropin hormones, which act through increasing cAMP in the ovary, did not result in the breakdown of membrane polyphosphatidylinositides in the present studies (Fig. 8-10). cAMP-inducing agents, CT, also did not have any effect on the hydrolysis of membrane phosphatidylinositide. Since this toxin enters cells via a ganglioside mediated mechanism, its onset of action is notoriously slow. A longer time may need for further study. On the other hand, gonadotropin hormones which are the major

hormones regulating ovarian functions, and LHRH which probably plays a local paracrine regulatory role, may interact with each other via their different intracellular signal pathways. Interactions between the adenylate cyclase pathway and phosphoinositide breakdown have been reported in various cell types. For instance, cAMP analogs have been shown to enhance the formation of polyphosphoinositides in sarcoplasmic reticulum preparations of rabbit heart and pig granulocytes (Enyedi et al., 1984; Farkas et al., 1984), but significantly inhibit norepinephrine induced inositol phosphate accumulation in FRTL-5 cells (Bone et al., 1986). Prostacyclin, which stimulates cAMP accumulation, has been shown to block thrombin stimulated PI turnover (Watson et al., 1984). More recently, FSH has been shown to inhibit the serum stimulated accumulation of inositol phosphate, but FSH itself has no significant effect on the formation of inositol phosphate in Sertoli cells (Monaco et al., 1988). The present results (Fig. 9-11), however, are in contrast with a previous report which showed that LH stimulated inositol phosphate formation in rat granulosa cells (Davis et al., 1986). The discrepancy between the previous and present studies cannot be easily explained. A possible reason could be the different research approaches undertaken. For example the long exposure of the cells in Davis's study to LH may facilitate the synthesis of membrane phosphoinositides.

In response to LHRH, other products of membrane inositol lipid hydrolysis such as DG were also detected in the present study (Fig. 6-8 and 10). Geison et al. (1976) found that the

sn-2 position of phosphatidylinositides was rich in AA. This knowledge was used in the present study to determine the effect of LHRH on DG production by labelling granulosa cells with [^{3}H]AA. The production of [^{3}H]DG was then measured. According to the time response study, LHRH-induced DG formation was observed as early as 15 sec. This time was very similar to that found for LHRH-induced increase in IP_3 (Ma and Leung, 1985; Davis 1986). Since the previous studies have demonstrated that LHRH only increases ^{32}P incorporation into phosphatidylinositol and phosphatidic acid, this similarity in time suggests that the increased level of [^{3}H]DG must most likely have resulted from phospholipase C hydrolysis of phosphoinositides (Naor and Yavin, 1982; Davis et al., 1983; Minegishi and Leung, 1985).

It is interesting to note that most of the [^{3}H]DG was recovered from the intracellular space (Fig. 7). Although LHRH did alter the amount of intracellular [^{3}H]DG, LHRH did not significantly change the level of DG in the medium. This finding was consistent with the character of DG as a membrane hydrophobic metabolite. Interestingly, a similar result has been observed with [^{3}H]IP_3 as well (Naor et al., 1986). These results may suggest that the DG and IP_3 formed by LHRH induction may have biological roles within the cells where they are produced rather than having an influence on other cells.

DG may play a potent role in the action of LHRH by activating PKC, which is a Ca^{2+} activated and phospholipid dependent protein kinase. The activity of this enzyme has been

demonstrated in the ovary (Noland and Dimino, 1986; Davis and Clark, 1983). DG and DG-like phorbol esters, i.e. TPA, stimulate PKC by reducing the amounts of Ca^{2+} and phospholipid required for activation (Nishizuka, 1984; Takai et al 1984). The dependence of PKC on phospholipid indicates that the enzyme activation may involve association of the enzyme with phospholipid-rich cell membranes. Similar to the effects of LHRH, both inhibitory and stimulatory effects of DG and TPA on ovarian steroidogenesis have been demonstrated, indicating that activation of PKC by endogenous diacylglycerols may serve as an amplifier of the LHRH-stimulated signal. (Welsh et al., 1984; Shinohara et al., 1985; Kawai and Clark, 1985). DG and TPA have also been shown to mimic the action of LHRH on LH release in the pituitary (Naor and Catt, 1981; Conn et al., 1985).

In addition to IPs and DG, the third compound that was measured in the present study was [^{3}H]AA (Fig. 6, 10 and 15). A previous study has demonstrated that LHRH causes an increase in the level of [^{3}H]AA release in the culture medium as early as 15 min after LHRH addition (Minegishi and Leung, 1986). The stimulatory effect of LHRH can be blocked by the concomitant presence of a potent LHRH antagonist. To evaluated the role of AA in the actione of hormones, it has also been observed that LHRH-stimulated LH release is closely coupled with the production of oxidized AA metabolites in the anterior pituitary (Naor and Catt, 1981; Snyder et al., 1983; Abou-Samra et al., 1986). On the other hand, it has also been suggested that AA itself rather than its metabolites may be a cellular regulator

of PRL secretion from GH_3 cells (Kolesnick et al., 1984). Since the intracellular concentration of free AA limits the synthesis of PGs and LTs, the demonstration of the increase in intracellular free AA is clearly important. In the present study with rat granulosa cells, LHRH stimulated [^{3}H]AA to increase by about 170% 5 min after the addition of LHRH (Fig. 6). The data thus indicate that LHRH action may be mediated by its induction of AA release. The mechanism of this LHRH-induced AA release in granulosa cells is, however, not clear. AA has indeed been made from inositol phospholipids through two consecutive reactions catalyzed by phospholipiase C followed by diacylglycerol lipase, which has been shown in platelets (Bell et al., 1979; Dixon and Hokin, 1984). LHRH has been found to cause an apparently selective depletion in the level of radiolabeled PI, suggesting that AA may be derived from inositol phospholipids (Minegishi and Leung, 1985). Thus the transient formation of DG as a result of agonist stimulated breakdown of membrane phosphoinositides represents a major pathway leading to liberation of AA for PGs and LTs synthesis. On the other hand, the release of intracellular free AA may also be due to the activation of PLA_2 which hydrolyzes AA from the sn-2 position of one or several phospholipids. The activation of PLA_2 is Ca^{2+} dependent. Verapamil, a calcium-channel blocker prevents both the enhancement of AA release and the depletion in the level of radiolabeled PI in rat granulosa cells. In addition, the omission of Ca^{2+} from the incubation medium could also diminish LHRH-induced [^{3}H]AA release in these

cells (Minegishi and Leung, 1985). These findings strengthen the concept that Ca^{2+} is required at a step before AA release, as suggested previously by other studies (Folkert et al., 1984; Forder et al., 1985; Naor and Catt, 1981). Additionally, $[Ca^{2+}]i$ mobilization induced by IP_3 could liberate AA from phosphoinositide via PLA_2 activation. However, neither the relationship between the two pathways, nor the relative amounts that each contributed to AA liberation was clearly understood in granulosa cells. Recently, another possible mechanism has been proposed suggesting that PLA_2 is itself regulated by both Ca^{2+} and DG. According to this mechanism, PLA_2 activity is suppressed by a lipocortin. Receptor activation by agonists leads to an increase in intracellular calcium and the concomitant production of DG, from the breakdown of membrane phosphatidylinositide. The activation of PKC by DG induces the phosphorylation of the lipocortin, suppressing its anti-PLA_2 activity, and in the presence of increased $[Ca^{2+}]i$, optimal activity of PLA_2 is evoked (Touqui et al., 1986). In the present study, the release of AA induced by the calcium ionophore A23187 was enhanced by concomitant treatment of granulosa cells with TPA (10^{-7}M) (Fig. 15), presumably related to a PKC activated mechanism of AA release in granulosa cells. A similar TPA mediated potentiation of the calcium ionophore induced AA release in human platelet has been reported (Volpi et al., 1985). However, the nature of this proposed mechanism has not been elucidated in granulosa cells, although it seems that Ca^{2+} plays a major role. The three possible pathways

involved in AA release are summarized in Fig. 16.

As mentioned earlier, phorbol esters mimicked the LHRH action on hormone production in ovary and this probably involves activation of the cellular PKC. In addition to its role in control of cellular secretion, PKC, at least in some cells, controls the sensitivity of the phosphoinositide-signaling pathway by regulating receptor function or the PIP_2 content of the cell membrane (Taylor et al., 1984; Cooper et al., 1985). Specifically, the activation of PKC by DG is capable of stimulating PIP_2 synthesis and could theoretically increase the amounts of IP_3 and the formation of DG in response to receptor occupation (De Caffoy de Courcells et al., 1984). In contrast to the stimulatory effect of PKC on PIP_2 synthesis, the same kinase may also exert a negative control on the synthesis of PIP_2 (Aloy et al., 1983). In addition, receptors and their ability to functionally couple to PLC can also be regulated by PKC activation. It has been observed that the activation of PKC may completely block the agonist induced IP_3 production by decreasing the number of receptors and regulating the coupling of receptors to PLC through a G protein (Cooper et al., 1985; Lynch et al., 1985). In the present study, treatment of granulosa cells with TPA (10^{-7}M) stimulated the basal inositol phosphate formation, but the combined treatment of granulosa cells with LHRH and TPA did not potentiate or attenuate inositol phosphate formation induced by LHRH alone (Fig. 13). The stimulatory action of TPA on inositol phosphate formation was specific, since an inactive form of the phorbol

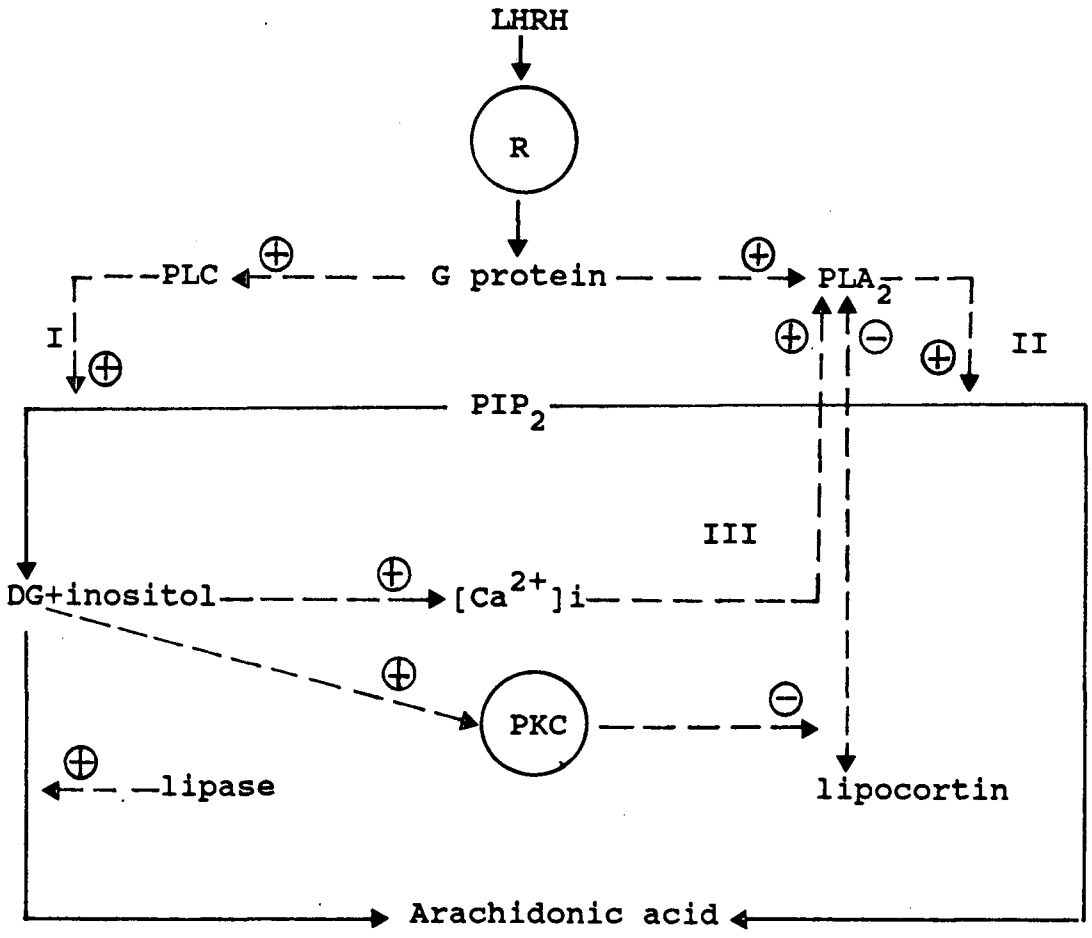

Fig. 16. Scheme showing proposed mechanism involved in arachidonic acid release.

ester, 4-alpha-phorbol 12, 13-didecanoate, did not alter the formation of inositol phosphate (Fig. 14). So far, the site of this action in granulosa cells was not clear. It has been demonstrated that TPA causes translocation of PKC from the cytosol to the cell membrane (Kraft et al., 1982). Rapid

redistribution of PKC activity during the onset of LH release in pituitary cells in response to LHRH (Nirota et al., 1985) suggests that the membrane localization of the active PKC may lead to the activation of phospholipase C and A_2 directly. Since PLC is the enzyme that catalyzes the hydrolysis of membrane phosphatidylinositide, it might be concluded that PKC activation is affecting, in some manner, the PLC hydrolysis of the phosphoinositides. However, it cannot be ruled out that PKC activation may decrease the hydrolysis of inositol phosphate to inositol, or increase the synthesis of PIP_2. As the source of membrane phospholipid is the same and the quantity of phosphatidylinositides is limited, the TPA induced inositol phosphate formation was overridden by the concomitant presence of LHRH (Fig. 13). Taken together, these data suggest that the activation of PKC plays a role in the formation of IP_3, DG and AA.

In summary, the interaction of LHRH with its plasma membrane specific receptors results in the rapid breakdown of membrane phosphoinositides, leading the production of IP_3, DG and AA. The subsequent changes of Ca^{2+} mobilization, PKC activation and the metabolism of AA may control the cellular secretion of granulosa cells. In addition, the activation of PKC and Ca^{2+} mobilization may also control the formation of IP_3, DG and AA by regulating receptors, PLC activity, and PIP_2 synthesis.

Chapter 3. Effect of LHRH on Cytosolic Free Calcium Ion Concentrations in Individual Granulosa Cells

I. Introduction

It has been documented that LHRH exerts direct actions on rat ovarian cells (Hsueh and Erickson, 1979; Hsueh and Jones, 1981; Clark, 1982; Hillensjo et al., 1982; Leung, 1985). The mechanism of actions of LHRH on the ovary is due to the stimulation of polyphosphoinositide breakdown in the cell membrane (Ma and Leung, 1985; Davis et al., 1986). Inositol 1,4,5-trisphosphate (IP_3), a product of hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP_2), has been proposed to induce intracellular Ca^{2+} mobilization (Berridge, 1984). In addition, products of phosphoinositide turnover may also be involved in regulating Ca^{2+} entry from the extracellular fluid (Berridge, 1987).

Many cellular functions such as cell movement, division, secretion and activation depend on changes in the free cytosolic calcium ion concentrations (Cheung, 1987). Calcium has also been demonstrated to be an important signal transduction agent in numerous tissues and cells (Rasmussen and Barrett, 1984).

To evaluate the role of calcium as an intracellular second messenger, quantitative measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) is required. The most popular method for measuring $[Ca^{2+}]i$ is to monitor the shifts in wavelength of fluorescent Ca^{2+} indicators when they bind Ca^{2+}.

These indicators are tetracarboxylic acid derivatives of the calcium chelator EGTA [ethylene glycol bis(ß-aminoethylether)-N,N'-tetraacetic]. A recent study on suspensions of granulosa cells using the calcium indicator dye, quin-2, measured only average free Ca^{2+} changes (Davis et al., 1986). This dye method was not designed to examine responses of individual cells and it is uncertain whether the increase of $[Ca^{2+}]i$ was due to an increased entry of Ca^{2+} across the cell membrane or to Ca^{2+} release from an intracellular site.

The recently developed fluorescent Ca^{2+} indicator, fura-2-acetoxy-methyl ester (fura-2AM) possesses fluorescent properties more appropriate for intracellular studies. This has increased the precision of the measurement of Ca^{2+} by reducing the effects of instrument drift, and more importantly has permitted the measurement of Ca^{2+} without determining the intracellular concentration of the dye. Moreover, because of the greater fluorescence intensity, the intracellular concentration of the dye can be reduced thus avoiding a calcium buffering effect. The selectivity of fura-2 for Ca^{2+} has also been slightly improved (Grynkiewicz et al., 1985).

Using fura-2, the present study examined: (1) the $[Ca^{2+}]i$ level in individual cells in primary cultures of dispersed granulosa cells; (2) the actions of LHRH on $[Ca^{2+}]i$ and (3) the contributions of intracellular and extracellular source(s) of Ca^{2+} in the LHRH-induced $[Ca^{2+}]i$ changes.

II. Materials and Methods

Preparation of animals and granulosa cells

Animals and granulosa cells were prepared as described in the Chapter 2.

Granulosa cells (10^5 cells/ml) were plated onto 18 mm diameter uncoated glass coverslips in 6-well culture dish and incubated in MEM containing 5% FBS. After 2 to 3 days of incubation at 37°C in an atmosphere of 5% CO_2 in air, cells were loaded with fura-2AM (Molecular Probes Inc., Eugene, OR), as described previously (Grynkiewicz et al., 1985).

Preparation and loading of the fura-2AM indicator

Fura-2AM was obtained in 1 mg quantities. The total amount was dissolved in 1 ml of chloroform and 50 µl aliquots were pipetted into 20 small plastic ampules. These ampoules were placed in a dessicator, and vacuum-dried for 3h. The dried aliquots were stored at -70°C.

For each culture used, 1 to 3 ml aliquots of Earl's Balanced Salt Solution (EBSS) for fura-2AM loading were pipetted into a plastic tube. The components of the EBSS were as follows: 117 mM NaCl, 1 mM $NaH_2PO_4.H_2O$, 5.6 mM glucose, 10 mM HEPES, 26 mM $NaHCO_3$, 5mM KCl, 0.8 mM $MgCl_2.6H_2O$ and 1.8 mM $CaCl.2H_2O$. Fura-2AM (50 µg) was dissolved in 50 µl of DMSO, to produce a stock solution (1mM). EBSS medium was pre-incubated at 37°C in a 5% CO_2 environment for at least 15 min to stabilize the pH at 7.4. Fura-2AM was added to EBSS while

vigorously agitating the medium on a mixer, producing a concentration of 10 μM fura-2AM (10 μl fura-2AM/ml of EBSS). EBSS (1 ml) containing fura-2AM was immediately added into a 6 well culture dish with another 1 ml of EBSS, and the granulosa cell culture was placed face-up in the well. Fura-2AM is hydrophobic and therefore penetrates the plasma membrane without difficulty. The cultured cells were then incubated with fura-2AM for 1 hour to allow the uptake to reach an equilibrium. Once inside the cell, cytosolic esterases cleave the acetoxymethyl groups from the indicator to release free fura-2 which is impermeable and therefore trapped inside the cells. At the end of the fura-2 "loading" incubation, cells were rinsed by placing in a dish of fresh EBSS (2 ml) for a further half-hour to wash out excess fura-2AM.

Fluorescence Measurement

Individual coverslips were mounted face-down onto a laminar flow-through chamber (volume 350 μl). Silicone grease was used to complete a water-tight seal and the chamber inserted into a stainless steel holder and the entire assembly mounted onto the stage of a Zeiss Jenalumar microscope equipped with epifluorescence detector. The light souce was a 200 Watt mercury arc lamp powered by a DC power supply. The light was first passed through one of three differential interference filters (350, 365 or 380 nm, bandwidths = 10 nm) mounted in a turret which could be rotated by a computer-controlled stepping motor. The light was then passed through a 410 nm dichroic

mirror and a 100x apochromatic oil immersion lens with a numerical aperture of 1.4 and an adjustable diaphragm to reduce the light intensity. A field diaphragm in the light path prior to the dichroic mirror was used to reduce the area of illumination to the size of a single granulosa cell. All fluorescent light passed back through the dichroic mirror and a 450 nm band pass filter to reduce background fluorescence. The emitted fluorescence taken at 350 nm (indicator fluorescence increased maximally with Ca^{2+} binding) and 380 nm (decreased with Ca^{2+} binding) was deflected either to the eyepieces or to a camera port. Onto the camera port was mounted a photomultiplier tube which was used to convert the fluorescence into DC voltage. This voltage was then converted to digital form by an Analogue-II Digital Converter in the computer. Measurements of fluorescence ratios were corrected for background and obtained on a 1.8 or 5 sec time base. The measurements were made at room temperature with low chloride EBSS (0.8 mM $MgSO_4.2H_2O$, 2.7 mM K_2SO_4, 117 mM Isethionate, 26 mM $NaHCO_3$, 1 mM $NaH_2PO_4.H_2O$, 5.6 mM glucose, 10 mM HEPES and 1.8 mM $CaCL_2.2H_2O$) constantly flowing at a rate of 4 ml/min throughout the experiment.

Cellular location of entrapped fura-2

Fura-2 was found to be uniformly distributed throughout the cytosol and nucleus of granulosa cells. The responsiveness of the fura-2 to changes in cytosolic $[Ca^{2+}]i$ was confirmed by direct injection into the laminar flow chamber of 50 µl of 5 µM

Br-A23187, a non-fluorescent calcium ionophore (HSC Reseach Development Corporation, Toronto, Canada). The cells could be used for up to 4 to 5h after loading with only minimal signs of leakage of fura-2.

Calculation of cytosolic calcium concentration

The cytosolic calcium concentration was calculated using the following formula (Grynkiewicz et al., 1985)

$$[Ca^{2+}]i = kd \times \beta \times R-R_{min}/R_{max}-R$$

Where: kd = the equilibrium dissociation constant for the association of fura-2 with cytosolic free calcium: 224 μM.

ß = ratio of the values: the fluorescence intensity at 380nm with zero $[Ca^{2+}]$/380nm with infinite $[Ca^{2+}]$.

R = experimentally determined ratio of the fluorescence intensity at 350nm/380nm

R_{min}= ratio of the values: the fluorescence of intensity at 350nm/380nm with zero $[Ca^{2+}]$.

R_{max}= ratio of the values: the fluorescence of intensity at 350nm/380nm with infinite $[Ca^{2+}]$.

For the present study, ß =10.07; R_{min} = 0.51; R_{max} = 4.83 were determined using the same granulosa cell cultures. At least 50 nM $[Ca^{2+}]i$ change was considered significant.

Calibration of the system

Each of the two cultures was first placed in a measuring chamber with standard EBSS (1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}) and

$[Ca^{2+}]i$ was determined in 20 to 40 cells in each culture. This was done to assess the health of the culture and to provide a control for further calibration. After measuring control values, one culture was rinsed with EBSS containing 5 mM EGTA and no Ca^{2+}. It was then placed onto a chamber which was filled with Ca^{2+}-free EBSS containing 5 mM EGTA and Br-A23187 (10 uM). $[Ca^{2+}]i$ measurements were taken immediately and after 15 min. After the second culture coverslip was measured for control values, the cells were immediately transferred to a chamber with standard EBSS containing Br-A23187. $[Ca^{2+}]i$ values were measured immediately (within seconds), as extracellular Ca^{2+} quickly entered the cell. ß, R_{min} and R_{max} constants were then calculated from the averages of the values obtained from these experiments.

Reagents

LHRH was purchased from Sigma. A potent LHRH antagonist, Ac-D-Nal $(2)^1$, 4 Cl-D-Phe^2, D-Trp^3, D-Ala^{10}-LHRH, was obtained as a gift from Dr. M.V. Nekola of Tulane University. Ovine LH (oLH-25), ovine FSH (oFSH-16) and pregnant mare's serum gonadotropin were obtained from the NIDDK and National Hormone and Pituitary Program (University of Maryland School of Medicine). Other chemicals were obtained from Sigma.

III. Results

Rapid and transient effects of Br-A23187 on cytosolic calcium

After loading with fura-2, granulosa cells were challenged by injection into the flow through-chamber of the calcium ionophore Br-A23187. Fig. 17 shows a representative example of the effect of a 50 µl of injection of Br-A23187. There was a rapid and transient increase in $[Ca^{2+}]i$ 18 sec after the injection of Br-A23187 with a peak value of cytosolic $[Ca^{2+}]i$ approximately 8-10 fold above the resting level. This time delay was due to the time required for the injection volume to flow to the observed cell and the relatively slow incorporation of the ionophore into the membrane of the cells.

LHRH-induced transient increase in cytosolic calcium

Each of the 115 rat granulosa cells from 27 different preparations were treated with LHRH. The average resting level of $[Ca^{2+}]i$ of these cells was 96.7 ± 2.9 nM. Eighty-six cells of the total 115 responded to LHRH. Table I illustrates that the hormone concentration required to increase $[Ca^{2+}]i$ was in the range of 10^{-9}M to 10^{-5}M. LHRH at 10^{-5}M increased $[Ca^{2+}]i$ in all of the cells which were sensitive to this hormone. The cells which did not respond to LHRH were sensitive to the calcium ionophore A23187 or Angiotensin II.

Fig. 18 shows a representative example of LHRH-induced rapid and transient $[Ca^{2+}]i$ alteration in a single rat granulosa cell. The determinations of $[Ca^{2+}]i$ were made at 1.8

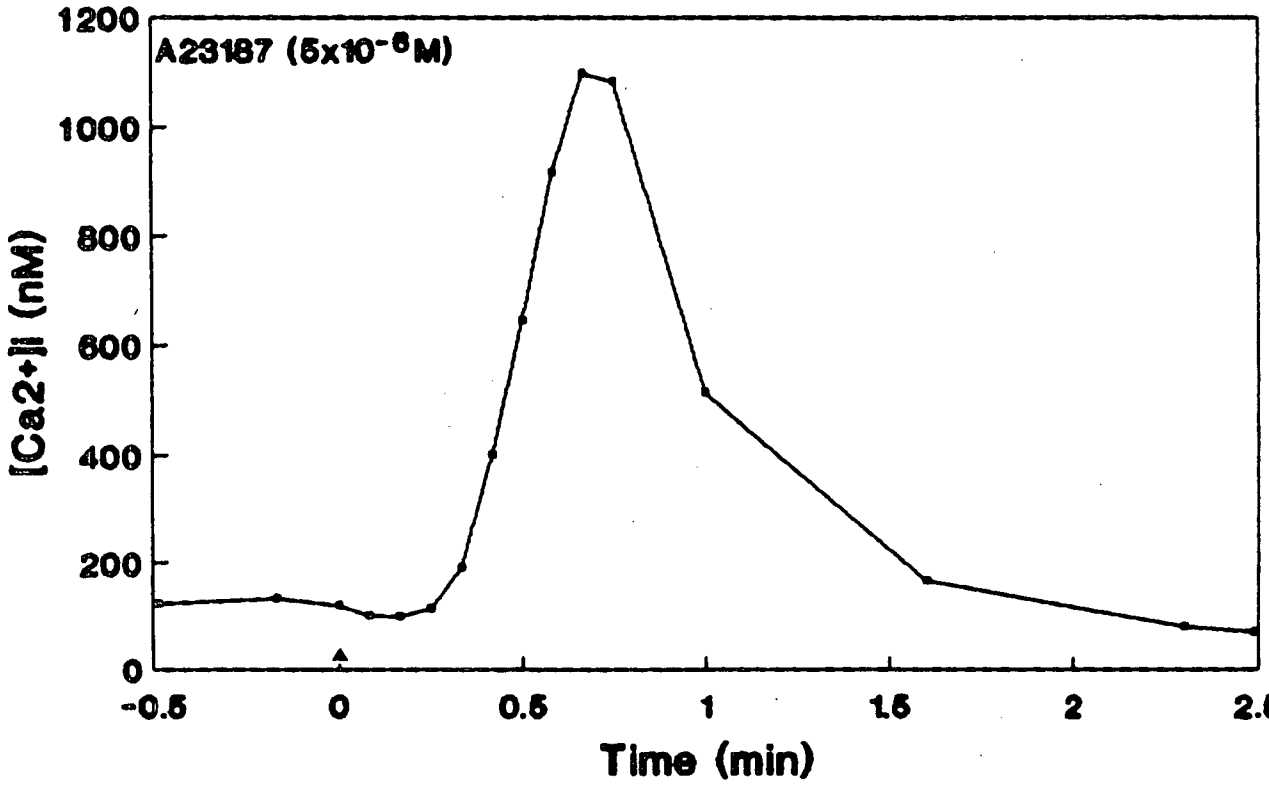

Fig. 17. Br-A23187 induced rapid and transient increase in cytosolic calcium. Coverslips with fura-2 loaded granulosa cells were mounted on a specially designed laminar flow-through chamber at room temperature. At the time (0 time) indicated by the symbol (▲), 50 µl of A23187 ($5x10^{-6}$M) was injected. The resulting images were measured by fluoroscence microscope microcomputer system at a 5 sec base (12 recording per min). Similar results were obtained from 9 individual cells in 6 separate experiments.

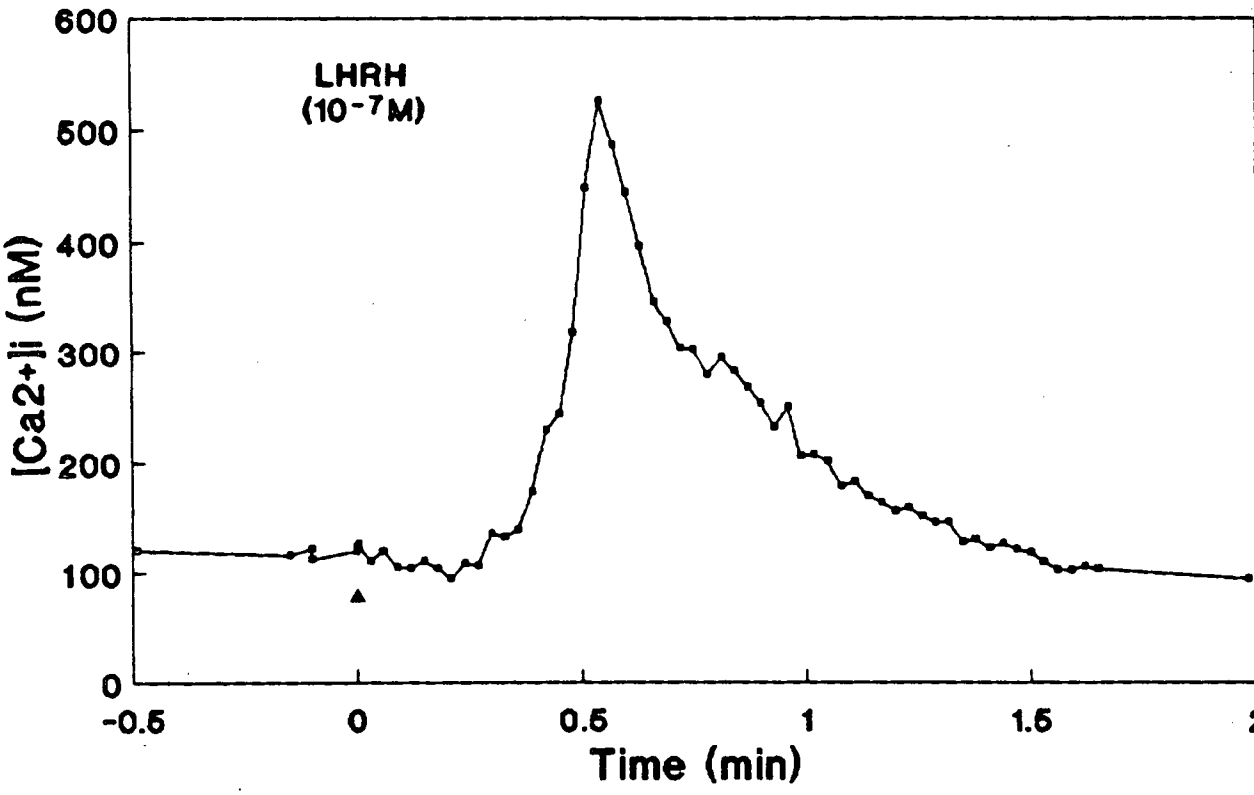

Fig. 18. LHRH induced rapid and transient increase in cytosolic calcium. At the time (0 time) indicated by the symbol (▲), 25 µl of LHRH was injected. The resulting images were measured at a 1.8 sec base (33 recording per min). The other experimental conditions were the same as those described in the legend of Fig. 17. Similar results were obtained from 21 individual cells of 8 experiments.

sec intervals. The average latency of the intracellular calcium response after the injection of LHRH was 21 ± 0.09 sec (n=70) and the average peak value induced by different doses of LHRH is shown in Table II. The amplitudes of the $[Ca^{2+}]i$ increase induced by the different doses of LHRH were not significantly different from each other. Within 84 ± 3 sec (n=64) after LHRH stimulation, $[Ca^{2+}]i$ returned to the resting level.

<u>The blockade of LHRH-induced cytosolic calcium alteration by LHRH antagonist</u>

To determine whether or not a receptor-mediated mechanism was involved in the action of LHRH on $[Ca^{2+}]i$, the effect of a potent LHRH antagonist was examined (Fig. 19). In each of 10 cells, an initial injection of 25 µl of 10^{-6}M LHRH resulted in a rapid and transient increase of $[Ca^{2+}]i$. LHRH antagonist (25 µl of a 10^{-5}M solution) was then injected into the cell chamber. The LHRH antagonist by itself had no direct effect on the resting $[Ca^{2+}]i$. On the other hand, two subsequent injections of LHRH, at 2 and 4.5 min following the administration of the LHRH antagonist, failed to increase $[Ca^{2+}]i$. These cells nonetheless still responded to Br-A23187 following the treatments with LHRH and LHRH antagonist.

Table I. lowest hormone concentrations required to initiate a change in cytosolic calcium in granulosa cells.

LHRH	Cell number
10^{-9}M	3
10^{-8}M	3
10^{-7}M	21
10^{-6}M	37
10^{-5}M	22
No response	29
Total	115

Table II. Average peak value of $[Ca^{2+}]i$ induced by different doses of LHRH.

LHRH	$[Ca^{2+}]i$ Change (fold)
10^{-7}M	4.97 ± 0.69 (n=21)
10^{-6}M	4.54 ± 0.32 (n=37)
10^{-5}M	4.51 ± 0.55 (n=22)

Existence of a subpopulation of granulosa cells: $[Ca^{2+}]i$ changes induced by different hormones

As illustrated in Fig. 20 (panel A), injection of 10^{-6}M LHRH caused a rapid and transient increase in $[Ca^{2+}]i$, whereas two injections of Ang II at 10^{-5}M and 10^{-4}M, respectively, did not affect the resting level of $[Ca^{2+}]i$ in the same cell. However, LHRH-induced $[Ca^{2+}]i$ alteration was not influenced by Ang II and an increase in $[Ca^{2+}]i$ induced by LHRH was observed after Ang II. In contract, the different result was observed from different individual granulosa cells. Fig. 20 (panel B) shows a representative example of Ang II (10^{-5}M) induced increase in $[Ca^{2+}]i$. However, the same cell did not respond to LHRH (10^{-5}M).

Desensitization of $[Ca^{2+}]i$ response induced by continuous exposure to LHRH

The upper panel of Fig. 21 shows a representative example of the cytosolic calcium increase stimulated by 3 separate injections of 25 µl of 10^{-6}M LHRH. The interval between the injections was 5 min. The increase in $[Ca^{2+}]i$ induced by these consecutive injections of LHRH reached similar maximum amplitudes. This pattern was seen in each of 14 cells, albeit the peak $[Ca^{2+}]i$ responses of the cells to the same dose of LHRH varied between 250 to 600 nM. The lower panel of Fig. 21 illustrates another representative example of $[Ca^{2+}]i$ alterations induced by repeated doses of 25 µl of 10^{-6}M LHRH given at intervals of less than 2 min. A gradual decrease in

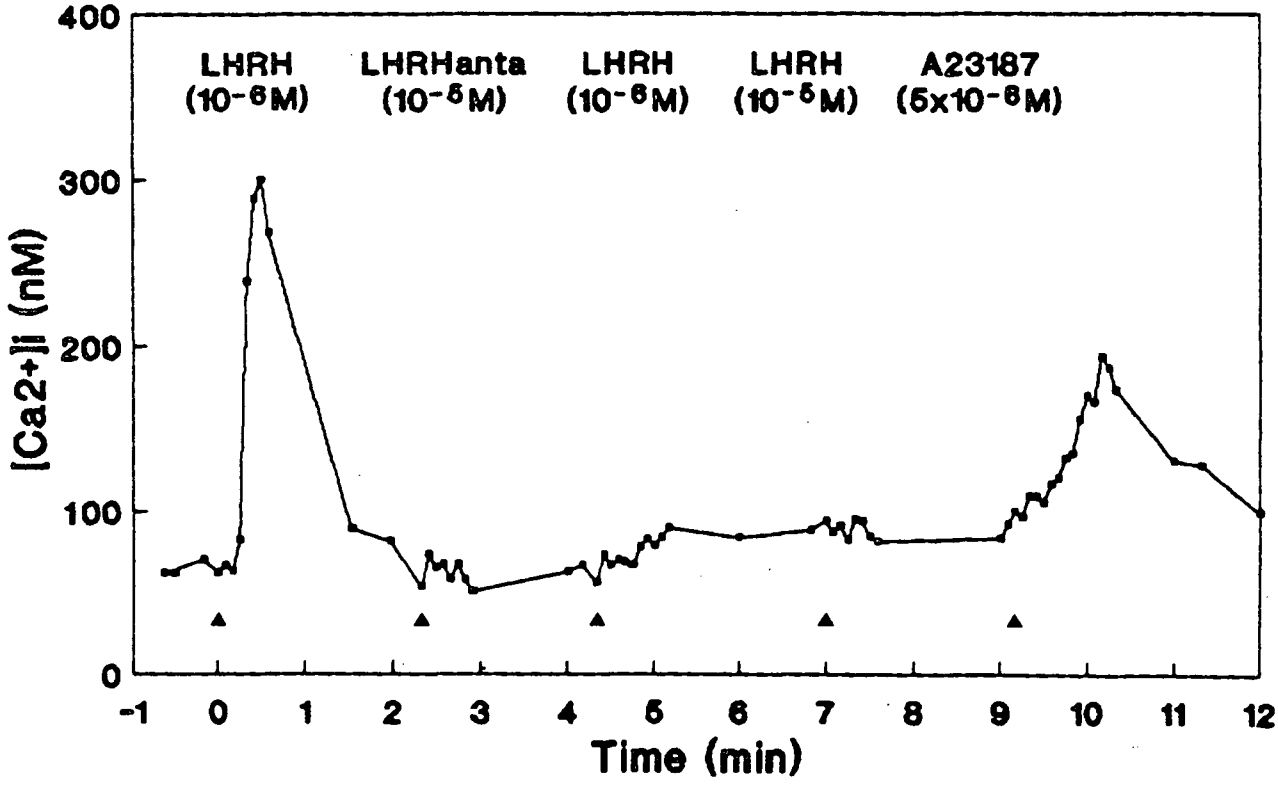

Fig. 19. The blockade of LHRH-induced cytosolic calcium alteration by LHRH antagonist. The experimental conditions were the same as those described under the legend of Fig. 17, but with 25 μl injections of LHRH, LHRH antagonist (LHRH anta), or Br-A23187 at the times indicated by (▲). Similar results were obtained from 10 individual cells of 10 experiments.

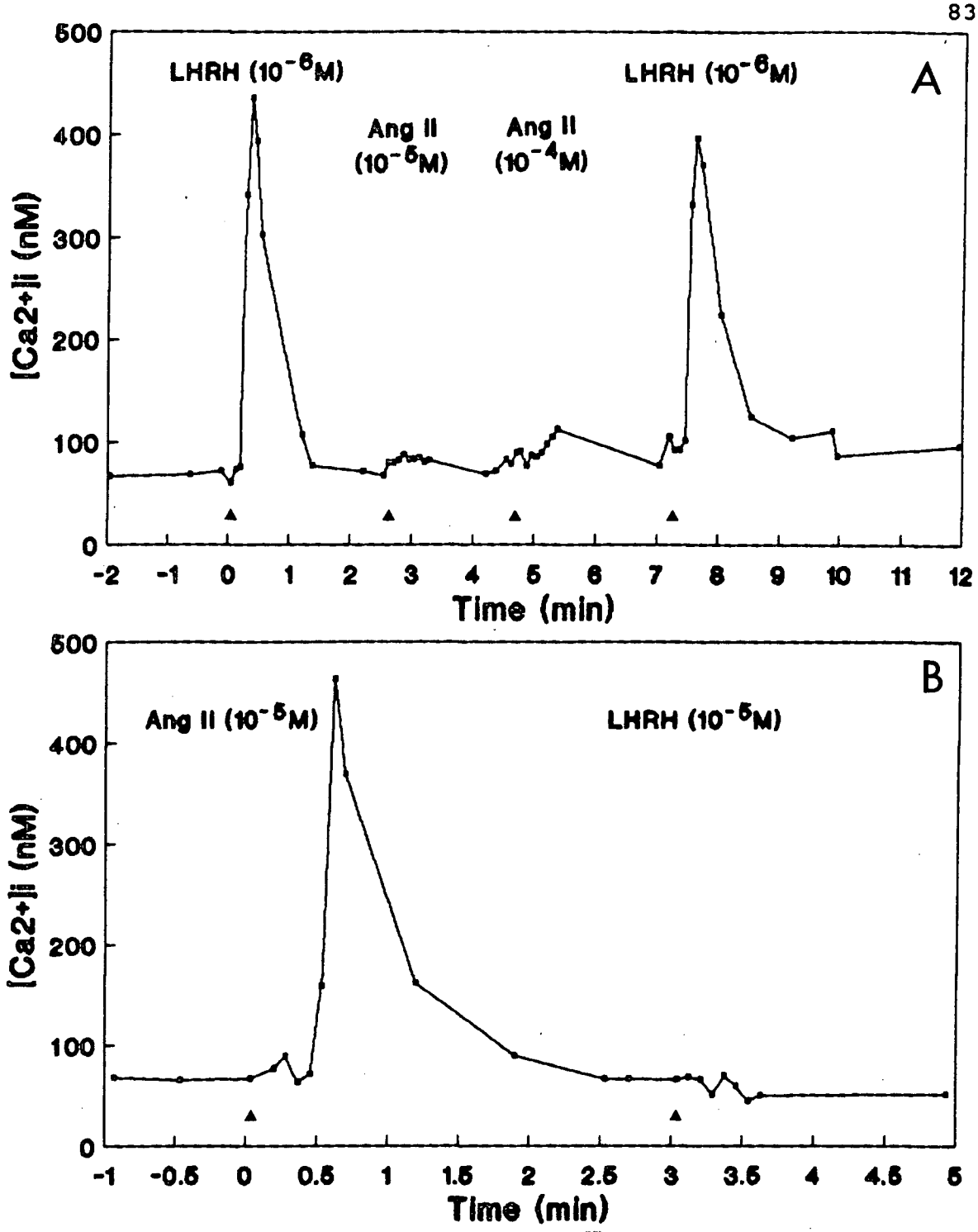

Fig. 20. Ca^{2+} Existence of subpopulations of granulosa cells: $[Ca^{2+}]i$ changes induced by LHRH and Ang II. Upper panel and lower panel show the representative examples of the cells responded to either LHRH or Ang II, respectively. (▲) indicates the injection of LHRH or Ang II. Similar results were obtained from 11 individual cells in 4 different experiments.

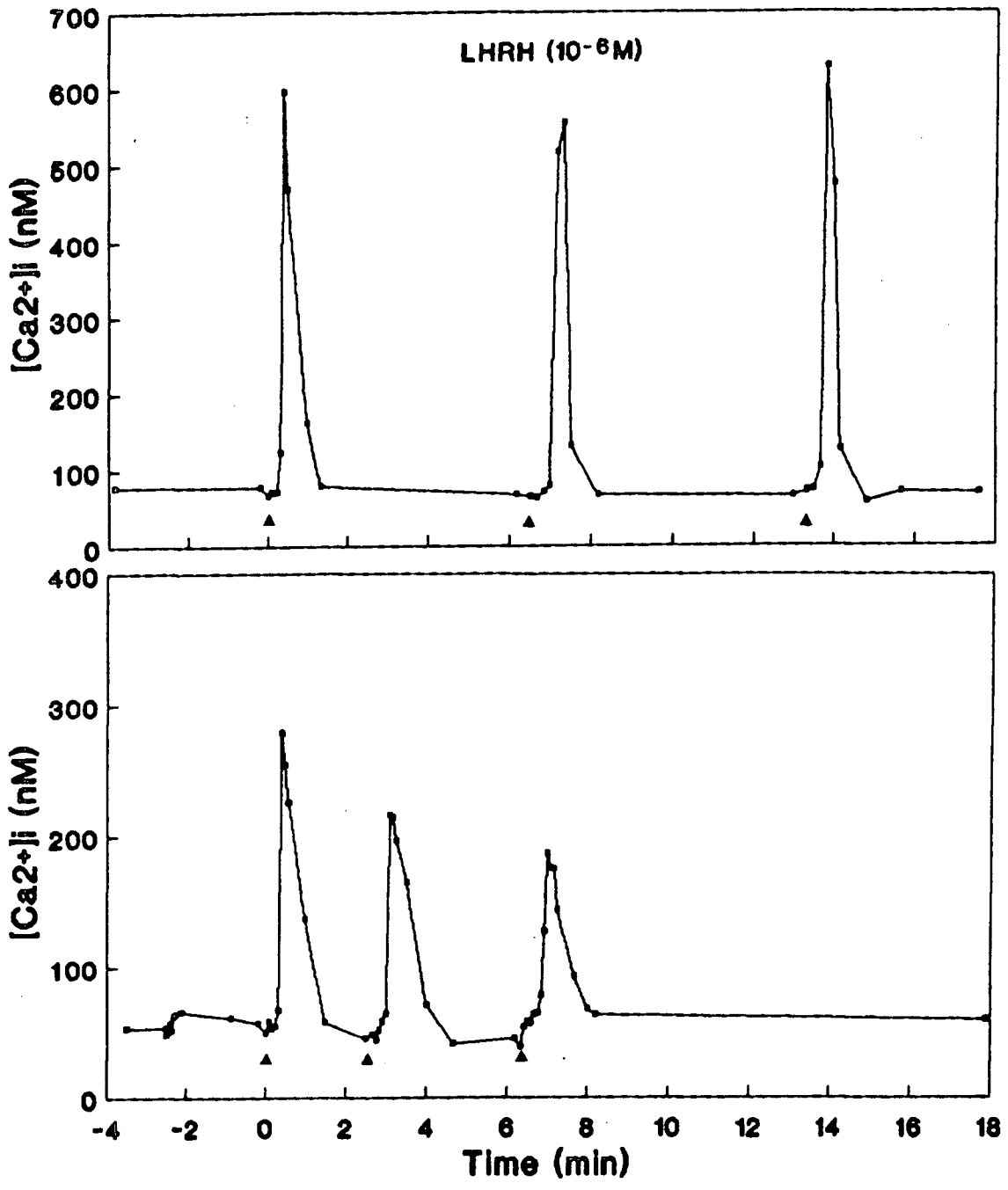

Fig. 21. Increase in $[Ca^{2+}]i$ induced by separate injections of LHRH to two individual granulosa cells. The upper panel shows the cytosolic calcium increase stimulated by 3 separate injections of LHRH. The lower panel shows a gradual decrease in the amplitude of $[Ca^{2+}]i$ induced by LHRH at intervals of less than 2 min. Similar results were obtained from 14 individual cells in 5 separate expriments.

the amplitude of $[Ca^{2+}]i$ could be seen. A striking example of desensitization induced by LHRH is shown in Fig. 22. In this experiment a granulosa cell was perifused continuously with 10^{-7}M LHRH for 10 min. The perifusion of LHRH caused a transient increase in $[Ca^{2+}]i$ which was not unlike the $[Ca^{2+}]i$ change induced by a pulse injection of LHRH. However, the increase in $[Ca^{2+}]i$ returned to the resting level despite the continued presence of LHRH. Furthermore, a pulse injection of 25 µl of 10^{-5}M LHRH 5 min after the initiation of the LHRH infusion period failed to increase $[Ca^{2+}]i$. In contrast, following the cessation of the infusion and after a wash period of 8 min, the same injection of 10^{-5}M LHRH resulted in a transient increase in $[Ca^{2+}]i$, albeit to a lesser amplitude than the initial effect of 10^{-7}M LHRH.

Effect of different doses of LHRH on $[Ca^{2+}]i$

Fig. 23 shows the change of $[Ca^{2+}]i$ induced by different doses of LHRH in a single granulosa cell. The cell was treated with sequential injections of LHRH from 10^{-8}M to 10^{-4}M. The interval between the injections was at least 10 min to avoid any possible desensitization. No significant difference in peak levels of $[Ca^{2+}]i$ was observed following the injection of different doses of LHRH in single cells, or when the response of different cells was analyzed together (Table II).

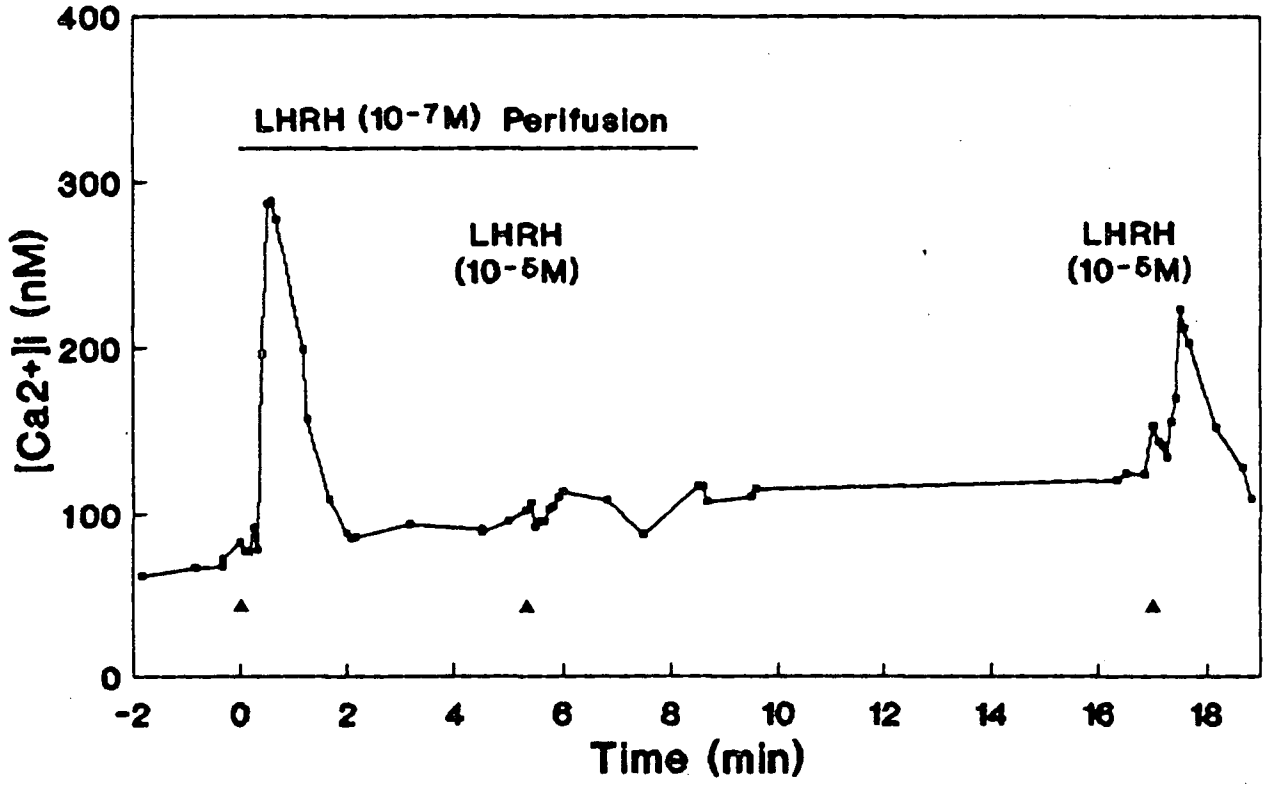

Fig. 22. Desensitization of $[Ca^{2+}]i$ response induced by continuous exposure to LHRH. First (▲): LHRH (10^{-7}M) perifusion for 10 min; second and third (▲): 25 µl LHRH (10^{-5}M) injections. Similar results were obtained from 6 individual cells of 4 experiments.

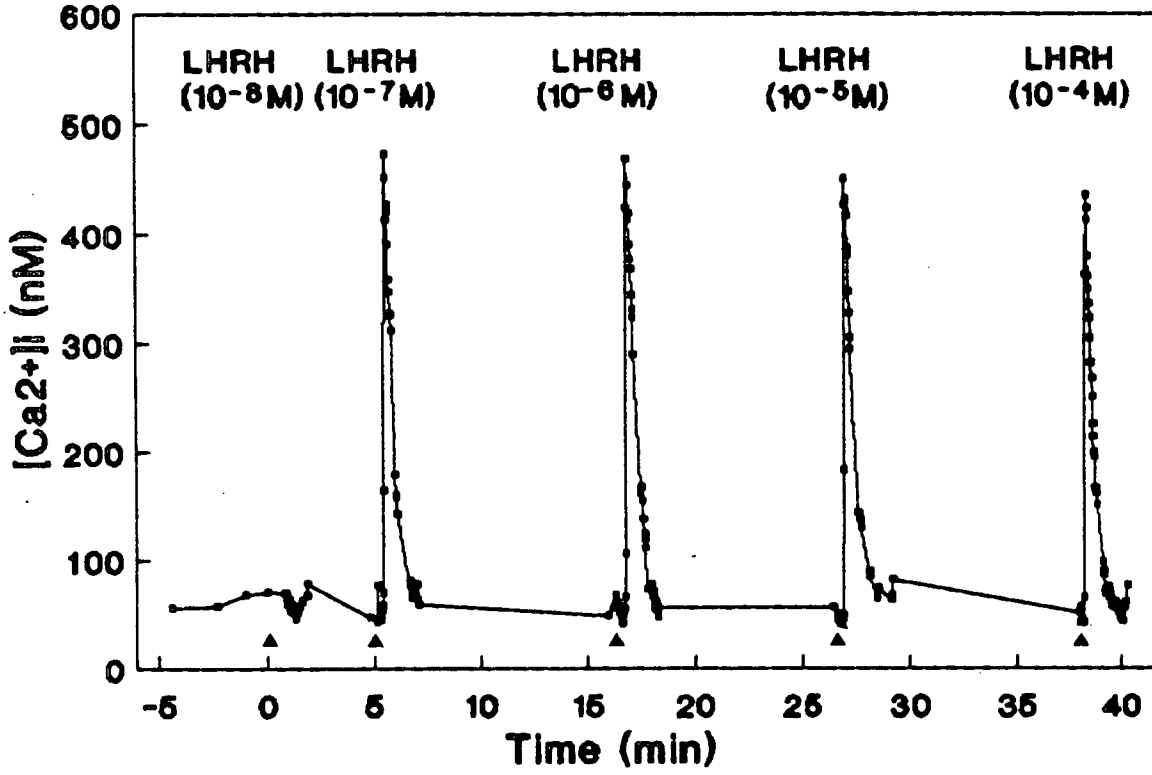

Fig. 23. Alterations in $[Ca^{2+}]i$ induced by different doses of LHRH. The cell was treated with sequential injections of LHRH from 10^{-8} to 10^{-4}M and no significant difference in peak level was observed. Similar results were obtained from 11 individual cells in 4 separate experiments. (▲) indicates the injection of LHRH.

Influence of Ca^{2+} free medium on $[Ca^{2+}]i$ alteration

To determine the influence of Ca^{2+} free medium on $[Ca^{2+}]i$ alteration, granulosa cells were perifused with Ca^{2+} free EBSS following the rapid and transient increase in $[Ca^{2+}]i$ induced by LHRH ($10^{-6}M$) in normal Ca^{2+} EBSS. Fifteen minutes after the Ca^{2+} free EBSS perifusion, two sequential injections of LHRH ($10^{-6}M$ dissolved in Ca^{2+} free EBSS) were made and $[Ca^{2+}]i$ did not increase in response to LHRH. Interestingly, granulosa cells responded to LHRH ($10^{-6}M$) normally again after 7 min of washing with normal EBSS. Fig 24. shows a representative example of 14 cells tested in the similar condition. The washing time required to establish a completely non-responsive condition in Ca^{2+} free EBSS varied from 8 min to 20 min in the different granulosa cells studied.

Role of intracellular Ca^{2+} in LHRH-induced $[Ca^{2+}]i$ alternation

The source(s) of calcium which contributed to the increase of $[Ca^{2+}]i$ induced by LHRH was further examined. As illustrated in upper panel of Fig. 25, after the initial increase of $[Ca^{2+}]i$ induced by $10^{-6}M$ LHRH in normal EBSS, granulosa cells were perifused with Ca^{2+} free EBSS medium. After washing with Ca^{2+} free EBSS for 8 min, the injection of LHRH ($10^{-6}M$) resulted in a rapid and transient increase of $[Ca^{2+}]i$ but with a significantly decreased amplitude (about 35% of the amplitude of $[Ca^{2+}]i$ increase in normal EBSS medium). In addition, a notable decrease of basal $[Ca^{2+}]i$ was also observed after the first injection of LHRH in Ca^{2+} free EBSS,

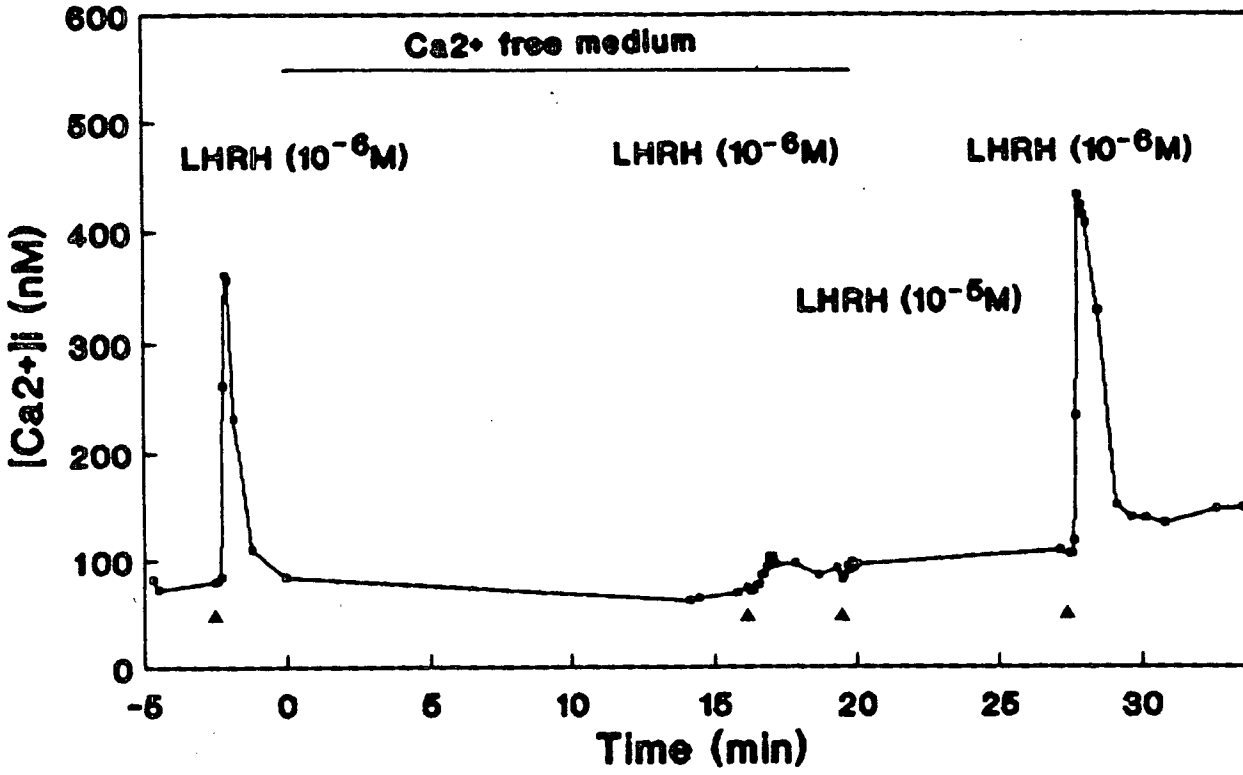

Fig. 24. Depletion of intracellular Ca^{2+} in calcium free medium. After the initial increase of $[Ca^{2+}]i$ induced by LHRH in normal medium, the cells were perifused with Ca^{2+} free medium. Fifteen minutes after the Ca^{2+} free medium perifusion, $[Ca^{2+}]i$ did not increase in response to LHRH. The cell responded to LHRH again after washing with normal medium. Similar results were obtained from 9 individual cells in 5 experiments. 0 time indicates the entry of calcium free medium.

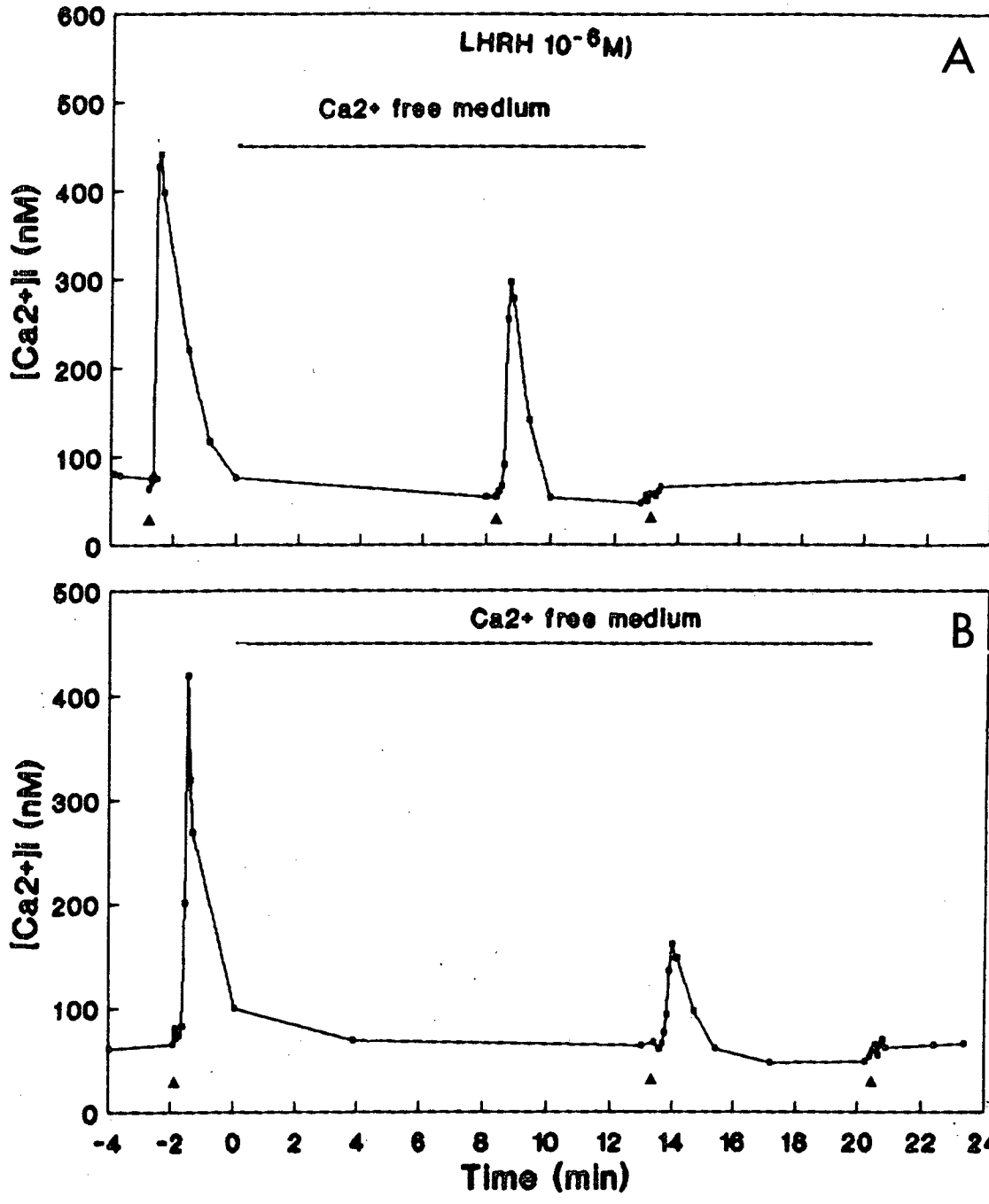

Fig. 25. LHRH accelerated $[Ca^{2+}]i$ depletion in Ca^{2+} free medium. The first (▲) indicates the injection of LHRH in normal medium; the second and third (▲) indicate the injections of LHRH in Ca^{2+} free medium (upper and lower panel). Similar results were obtained from 6 individual cells in 4 experiments. 0 time indicates the entry of calcium free medium.

and $[Ca^{2+}]i$ did not subsequently respond to the injection of LHRH at 13 min.

When LHRH (10^{-6}M) was injected at 13 min instead of 8 min after washing with Ca^{2+}-free EBSS, a transient increase of $[Ca^{2+}]i$ was observed, albeit with a smaller amplitude than that induced by LHRH at 8 min in Ca^{2+} free EBSS medium (Fig. 25).

Role of extracellular Ca^{2+} in LHRH-induced alteration of $[Ca^{2+}]i$

Fig. 26 shows the increases in $[Ca^{2+}]i$ in a single granulosa cell following the injection of LHRH at 0 time, followed by continuous washing the cell with Ca^{2+} free EBSS. After entry of Ca^{2+} free EBSS medium, LHRH-induced $[Ca^{2+}]i$ change was first decreased and eventually completely abolished. Subsequently, four separate injections of LHRH, which were dissolved in EBSS medium with 2 mM, 5 mM, 10 mM and 20 mM calcium, did not result in the change of $[Ca^{2+}]i$. LHRH caused the increase of $[Ca^{2+}]i$ again in the same cell following the perifusion of normal EBSS.

Comparison of gonadotropins with LHRH on $[Ca^{2+}]i$ alteration

Fig. 27 illustrates that when a single granulosa cell was stimulated by two separate 25 µl injections of 50 µg FSH, the $[Ca^{2+}]i$ was not altered. The injection 25 µl of LHRH (10^{-6}M) following FSH resulted in the expected increase in $[Ca^{2+}]i$. Similar results were obtained with 8 individual cells. In addition, as shown in Fig. 28, a granulosa cell which responded

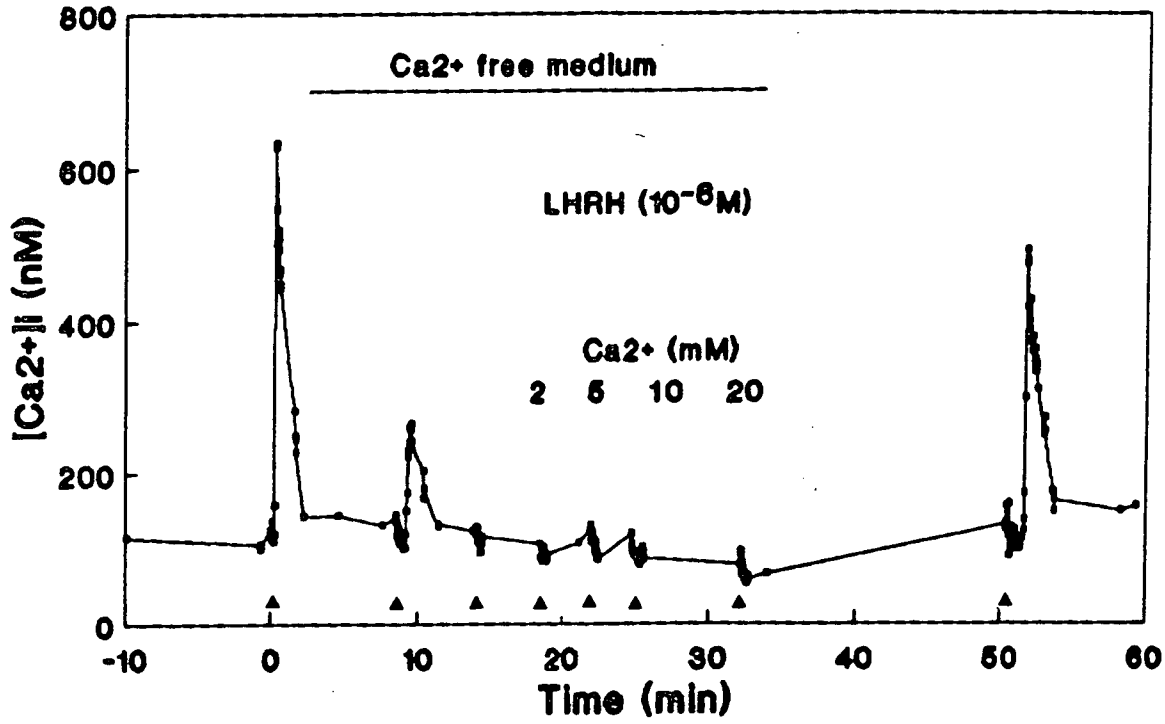

Fig. 26. Role of extracellular Ca^{2+} in LHRH-induced alteration of $[Ca^{2+}]i$. LHRH was 10^{-6}M for all the treatments. First (▲): LHRH injection in normal medium; second and third (▲): LHRH injections in calcium free medium; fourth to seventh (▲): LHRH plus different concentrations of Ca^{2+} injections in calcium free medium; eighth (▲): LHRH injection in normal medium. Similar results were obtained from 4 cells of 4 experiments.

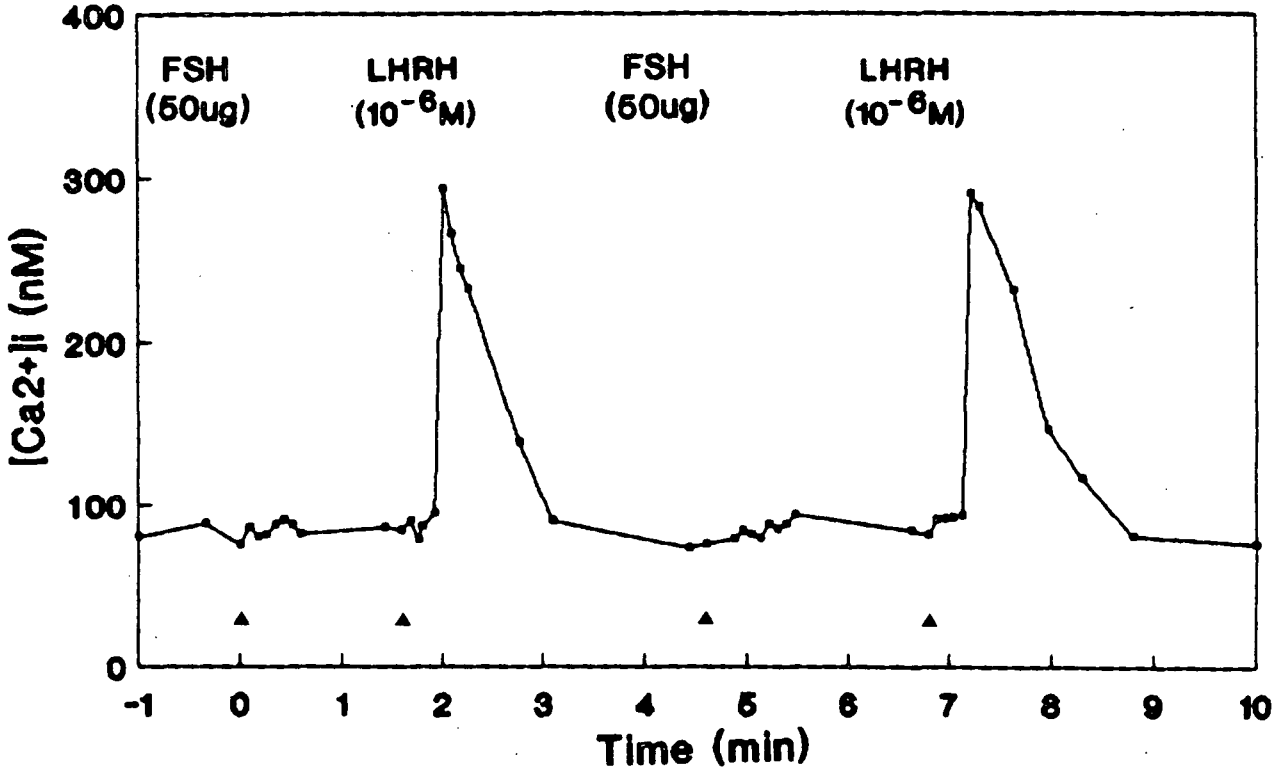

Fig. 27. Comparison of FSH with LHRH on $[Ca^{2+}]i$ alteration. LHRH resulted in rapid and transient $[Ca^{2+}]i$ alteration, whereas FSH had no effect. First and third ( ): 25 ul of injection of FSH; second and fourth ( ): LHRH. Similar results were obtained from 8 individual cells in 5 experiments.

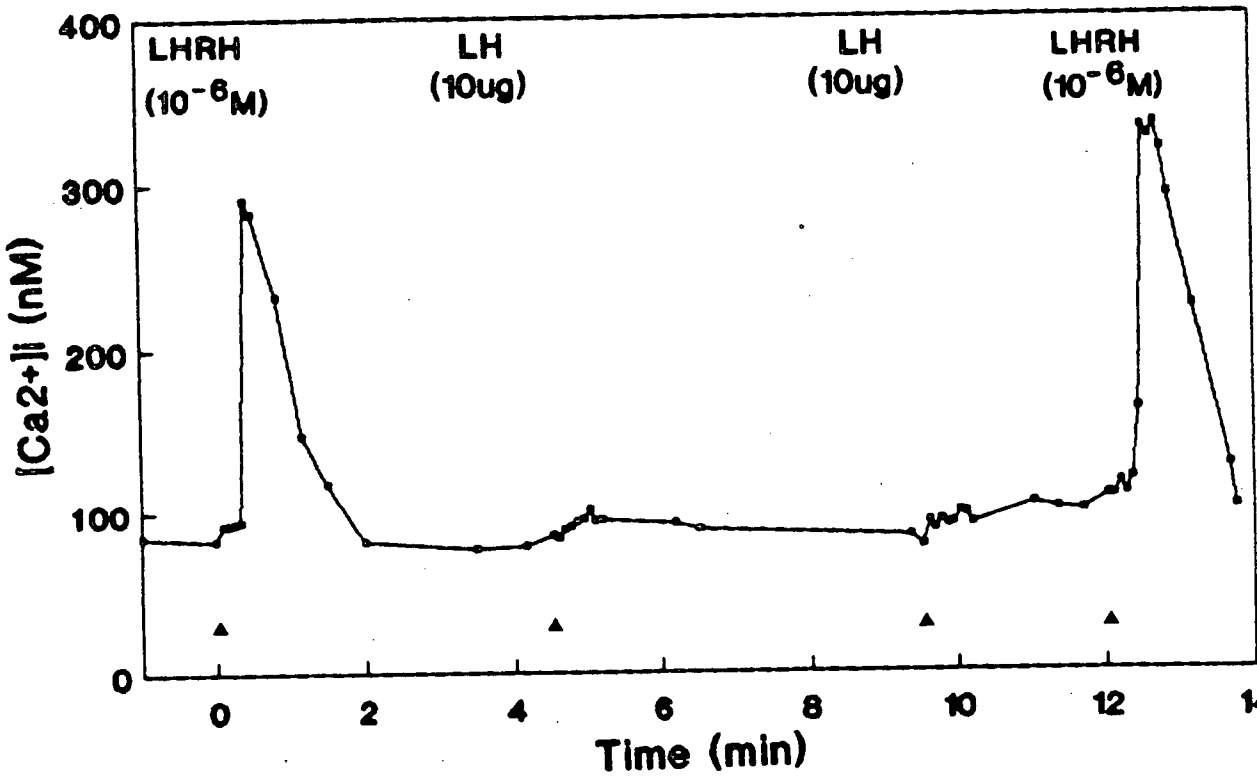

Fig. 28. Comparison of LH with LHRH on $[Ca^{2+}]i$ alteration. The cell which responded to LHRH did not respond to LH. Similar results were obtained from 8 individual cells in 4 experiments.

to LHRH (10^{-6}M) did not respond to 10 ug of LH, nor did the two separate injections of LH have any influence on the subsequent LHRH-induced increase in $[Ca^{2+}]i$. Identical results were seen in 7 other cells.

IV. Discussion

The calcium-sensitive fluorescent indicator, fura-2 has been used to study the effect of LHRH on the $[Ca^{2+}]i$ of individual rat granulosa cells. LHRH caused a rapid and transient increase in $[Ca^{2+}]i$ in the majority of cells tested (Fig. 17). Since an LHRH antagonist completely blocked the $[Ca^{2+}]i$ response of the cells to LHRH (Fig. 19), it could be concluded that the effects of LHRH on $[Ca^{2+}]i$ are mediated by its specific receptors.

However, the concentrations of LHRH required to produce a $[Ca^{2+}]i$ response varied considerably (10^{-9}M to 10^{-5}M) from cell to cell (Table I). This is not likely to have resulted from the design of the laminar flow-through chamber. It has been estimated that each dose of LHRH would only be diluted by no more than a factor of 2-5, depending upon the flow rate used and the position of the cell relative to the input of the chamber. Therefore, the varied concentrations of LHRH required by the individual granulosa cells may be due to the different threshold of the cells (see following discussion).

Most cells responded to LHRH in the range of 10^{-7}M to 10^{-5}M, but for any single cell there was no clear dose-related response to LHRH (Fig. 22; Table II). The lowest dose of LHRH

which resulted in an increase in $[Ca^{2+}]i$ appeared to yield a maximum response since higher LHRH concentrations given to the same cell did not result in additional increases in $[Ca^{2+}]i$. Thus, single granulosa cells seem to respond in an "all or none" fashion. Previous studies have, however, shown that LHRH and its agonists do produce dose-dependent stimulatory and inhibitory effect on progesterone production (Hillensjo et al., 1982; Knecht et al., 1985;). LHRH-induced arachidonic acid liberation from the cell membranes (Minegishi and Leung, 1985) and LHRH-stimulated inositol phosphate formation in rat granulosa cells are also dose-dependent (Ma and Leung, 1985; Davis et al., 1987). In addition, LH-induced $[Ca^{2+}]i$ alteration in bovine luteal cells has been shown to be dose-dependent as well (Davis et al., 1987). It is important to note, however, that in all these earlier studies populations of cells were used rather than the individual cells which were used in the present study. In this regard, it can be speculated that individual granulosa cells respond in an all or none fashion to LHRH, but that the threshold concentrations of LHRH required to stimulate different cells may differ. Hence when a mixed population of such cells is stimulated with LHRH, a dose response relation will be observed as progressively more cells "turn on". These data support the hypothesis of a quantal (i.e. all-or-none) response of hormonal control mechanism, since it has been suggested that all cells of a given type may not be equal in terms of hormonal responsiveness (Moyle et al., 1985).

The differences in cell-to-cell responsiveness were found to be randomly distributed in this study. The different minimum concentrations of LHRH required for initiating the cytosolic $[Ca^{2+}]i$ change may in part be related to the different functional states of the LHRH receptor in these cells.

While some individual granulosa cells responded to LHRH, others responded to different hormones such as Ang II (Fig. 20). Previous studies have shown that subpopulations of granulosa cells may exist with respect to differential sensitivity to FSH and vasoactive intestinal peptide (Kasson et al., 1985). In addition, PRL receptors have been shown to be more abundant in antral granulosa cells than in mural granulosa cells (Dunaif et at., 1982). The use of fura-2 microspectrofluorimetry techniques facilitated the investigation on the subpopulations of granulosa cells by allowing the study of individual granulosa cells. The present finding that different granulosa cells responded to different hormones may indicate that there are different subpopulations of granulosa cells which play different roles in response to different regulator-mediated ovarian functions.

One interesting observation made in this study was that with the decreasing time intervals between individual LHRH injections, the magnitude of the LHRH-stimulated increase in $[Ca^{2+}]i$ declined (Fig. 21). Furthermore, continuous exposure to a relatively low concentration of LHRH ($10^{-7}M$) resulted in desensitization of granulosa cells to higher ($10^{-5}M$) doses of

LHRH (Fig. 22). This may reflect the well-known down-regulation phenomenon of LHRH surface receptors, which may be due to massive internalization of the LHRH-receptor complex into endocytic vesicles (Hazum and Nimrod, 1982) and subsequent degradation of this complex. Peptide hormones, i.e insulin, LHRH (on gonadotrophes), and hCG, have been shown to induce motility, aggregation, and internalization of their receptors (Terris et al., 1979; Amsterdam et al., 1979; Hopkins and Gregory, 1977). In the present study, the decrease in receptor numbers may protect against intense stimulation by inappropriately high LHRH levels. On the other hand, this desensitization may be due to a receptor-mediated mechanism that is not related to the internalization of receptors. As it has been shown that only after 2 h of exposure, a significant proportion (10%) of the labeled hCG can be found in lysosome-like structures of rat granulosa cells, it is inferred that internalization is initiated within this time (Amsterdam et al., 1979). Moreover, in the present study, it was observed that after washing with fresh medium, the cells regained their responsiveness to LHRH in terms of $[Ca^{2+}]i$ (Fig. 22). The transient and reversible nature of this desensitization process may be more compatible with alternative mechanisms of desensitization, possibly at the level of signal transduction. It is interesting to note that, whatever the mechanism of this desensitization, fluctuations in LHRH levels may be more effective in stimulating a $[Ca^{2+}]i$ response in granulosa cells than a sustained elevation in LHRH concentration.

Recent studies have clearly shown that LHRH stimulates the formation of IP_3 in ovarian granulosa cells (Ma and Leung, 1985; Davis et al., 1986). IP_3 has been proposed as a mediator for intracellular Ca^{2+} mobilization (Nishizuka et al., 1984). Phosphoinositide turnover is also believed to be involved in the regulation of Ca^{2+} entry from the external environment (Berridge, 1987). Microinjection of IP_3 into some cells results in the $[Ca^{2+}]i$ mobilization and mimics calcium-dependent processes (Oron et al., 1985). Based on above observations, it appears that the $[Ca^{2+}]i$ changes stimulated by LHRH might be directly correlated to IP_3 formation. This hypothesis is supported by evidence obtained from the present and previous studies. LHRH antagonist can block LHRH-induced cellular responses including both IP_3 formation and $[Ca^{2+}]i$ mobilization (Ma and Leung, 1985; Fig. 19). Similar temporal relationships between LHRH-induced IP_3 formation and $[Ca^{2+}]i$ mobilization has been found by Davis et al. (1986).

Although LHRH resulted in the rapid and transient increase of $[Ca^{2+}]i$, the precise source(s) of Ca^{2+} which contributed to the cytosolic calcium alteration has to be resolved. In the present studies, the LHRH-induced changes in $[Ca^{2+}]i$ were completely abolished by washing with calcium free medium between 8 min to 20 min in 14 cells tested. Following re-perifusion with medium containing normal Ca^{2+}, the LHRH-induced increase in $[Ca^{2+}]i$ was again observed (Fig. 24). When the injection of LHRH was performed several minutes after the entry of Ca^{2+} free medium into the chamber, a significant

decrease in $[Ca^{2+}]i$ amplitude was observed as compared to the peak level of $[Ca^{2+}]i$ in the normal medium (Fig. 25). This decrease can be due to either the depletion of intracellular Ca^{2+} or the lack of Ca^{2+} in the extracellular fluid. Based on the above results, it can be estimated that the intracellular Ca^{2+} pool is depleted by passive diffusion when the external medium reaches a "Ca^{2+} free" condition (approx. 8 min). However, the complete depletion of $[Ca^{2+}]i$ was a gradual process, and a smaller increase of $[Ca^{2+}]i$ could still be observed in extracellular Ca^{2+} free condition, suggesting that LHRH does induce $[Ca^{2+}]i$ mobilization from an intracellular pool (Fig. 25). Ca^{2+} which is mobilized may easily diffuse into the extracellular solution. Therefore, with time the intracellular pool of Ca^{2+} would be exhausted, the cell would eventually lose responsiveness to LHRH. The LHRH-induced depletion of intracellular Ca^{2+} in Ca^{2+} free medium (Fig. 25) supports the concept that LHRH-induced increase of $[Ca^{2+}]i$ is, at least partially, from intracellular stores. The marked decline of the basal $[Ca^{2+}]i$ after the injection of LHRH in Ca^{2+} free medium further strengthens this notion (Fig. 25).

Since agonist-induced IP_3 is believed to be responsible for intracellular Ca^{2+} release, many studies have been made to elucidate which intracellular pool is responsible for the $[Ca^{2+}]i$ mobilization. The site at which IP_3 acts has been shown to be a ATP-dependent non-mitochondrial Ca^{2+} pool, probably ER. The experimental data obtained from previous studies have indicated that normal responses to IP_3 are

observed at free Ca^{2+} concentrations below the threshold for mitochondrial uptake or in the presence of mitochondrial inhibitors (Burgess et al., 1984). Whereas IP_3 releases Ca^{2+} when added directly to microsomes obtained from a variety of tissues, it fails to alter Ca^{2+} release from mitochondrial fractions (Streb et al., 1983; Prentki et al., 1984). Although subcellular fractionation studies have attributed a large portion of intracellular Ca^{2+} pool to mitochondria (Claret-Berthon et al., 1977), recent studies have demonstrated far less Ca^{2+} in mitochondria than in ER (Reinhart et al., 1984; Shears and Kirk, 1984). Electron probe X-ray microanalysis study of rapidly frozen liver also indicates that only 5% of cell Ca^{2+} is present in mitochondria, whereas 14-23% is within rough ER (Somlyo et al., 1985). The present results indicate that the intracellular Ca^{2+} pools are probably responsible for LHRH-induced increase of $[Ca^{2+}]i$. The relative importance of mitochondria and ER in granulosa cell cytosolic calcium regulation remains uncertain.

The possible contribution of extracellular Ca^{2+} to LHRH-induced increase of $[Ca^{2+}]i$ was next examined. LHRH dissolved in high concentration of Ca^{2+} (2 mM to 20 mM) was given to granulosa cells being perifused with Ca^{2+} free medium and in which the intracellular Ca^{2+} had been depleted. LHRH plus Ca^{2+} failed to evoke the increase of $[Ca^{2+}]i$ in the granulosa cell, suggesting that LHRH-induced $[Ca^{2+}]i$ alteration may not be due to the immediate Ca^{2+} influx across the cell membrane (Fig. 26). Alternatively, these results could suggest that even when

the intracellular Ca^{2+} pools are empty, the extracellular Ca^{2+} cannot quickly enter the cytosol. This is despite the existence of a gradient potential and the presence of LHRH to ensure the opening of the Ca^{2+} channels on the cell membrane. An early suggestion was that the Ca^{2+} content of intracellular pools regulated the entry of Ca^{2+} from the extracellular fluid; when the pools were empty, it was open to extracellular Ca^{2+} entry, and when the pools were filled, it was closed to the extracellular Ca^{2+} (Aub et al., 1982; Putney, 1986). Two phases of Ca^{2+} mobilization have been demonstrated in the previous studies. In the first phase, a release of intracellular Ca^{2+} in response to agonists, and in the second phase, entry of Ca^{2+} across the plasma membrane following the first phase (Kojima et al., 1985 Reynolds and Dubyak., 1985). In pituitary cells, LHRH elevates $[Ca^{2+}]i$ partly by releasing Ca^{2+} from intracellular pools and partly by triggering influx across the cell membrane. It has been shown that the elevation in $[Ca^{2+}]i$ induced by LHRH is composed of a rapid first phase followed by a prolonged increase in $[Ca^{2+}]i$ in the second phase (Clapper and Conn, 1985; Limor et al., 1987). Furthermore, Naor et al. (1988) have recently demonstrated that LHRH induces a rapid mobilization of intracellular Ca^{2+} pool, and a second component of Ca^{2+} influx via voltage sensitive and insensitive changes contributes to further elevation of $[Ca^{2+}]i$ in pituitary cells. Although the increase in $[Ca^{2+}]i$ induced by LHRH in individual granulosa cells did not obviously show two phases, that extracellular Ca^{2+} might also be involved in

LHRH-induced $[Ca^{2+}]i$ changes, with an initial intracellular Ca^{2+} mobilization triggering the influx of Ca^{2+}, cannot be excluded.

In addition, after washing the cells with medium containing normal Ca^{2+}, granulosa cells regained their response to LHRH (Fig. 24), which implied that a continued positive diffusion of Ca^{2+} from extracellular fluid was necessary to refill intracellular pools without a rapid change in cytosolic Ca^{2+}. Although it has been suggested that phosphoinositide turnover may be involved in the regulation of Ca^{2+} entry from extracellular fluid (Berridge 1984), experimental evidence indicates that either IP_3 or PKC regulates Ca^{2+} influx by a direct action at the plasma membrane (Streb et al., 1984; Cooper et al., 1985; Garrison et al., 1984). It appears that a decrease in the PIP_2 content of the cell membrane may inhibit the Ca^{2+}-ATPase and therefore cause an increase in $[Ca^{2+}]i$ (Berridge, 1982). This, however, is not a sufficient explanation for the rapid changes of $[Ca^{2+}]i$ induced by LHRH.

The effects of gonadotropins on granulosa cell $[Ca^{2+}]i$ were also investigated in the present study. Unlike LHRH, neither FSH nor LH, even at very high doses (10 ug of LH or 50 ug of FSH), had any effect on $[Ca^{2+}]i$ (Fig. 27; 28). In isolated bovine luteal cells, it has been reported that LH provokes a rapid increase in the accumulation of IP_3 and increase in $[Ca^{2+}]i$ (Davis et al., 1987). Treatment with LH, forskolin and cAMP may also cause $^{45}Ca^{2+}$ efflux in avian granulosa cells (Asem et al., 1987). In addition, exogenous

cAMP and forskolin have been shown to increase $[Ca^{2+}]i$ in Leydig cells (Sallivan and Cooke, 1986). It is possible that the action of gonadotropins on $[Ca^{2+}]i$ may be both species and cell specific. The present and previous studies demonstrate that, in rat granulosa cells, administration of LHRH leads to a rapid breakdown of inositol lipids and an increase in $[Ca^{2+}]i$, whereas gonadotropins consistently have no effect on either parameter (Ma and Leung, 1985).

A study of LHRH effect on $[Ca^{2+}]i$ in pituitary cells has shown that the increase in $[Ca^{2+}]i$ after LHRH was added to suspensions of gonadotroph-enriched pituitary cells could be correlated to the LH release (Naor et al., 1988). This suggests that $[Ca^{2+}]i$ plays an intermediary role when LHRH stimulates LH release from the pituitary. In the ovary, similar increases in $[Ca^{2+}]i$ may serve to modulate the stimulatory or inhibitory effects of LHRH on P_4 and PGE_2 accumulation (see Chapter 4).

In summary, the present study strongly indicates that LHRH causes a rapid and transient increase in cytosolic $[Ca^{2+}]i$ in individual rat granulosa cells. The action of LHRH on the cytosolic $[Ca^{2+}]i$ change is mediated by its specific receptors. By investigating individual granulosa cells, it is possible to demonstrate that different granulosa cells require different concentrations of LHRH to initiate what appears to be an "all or none" response and that different subpopulations of granulosa cells may also exist. Another interesting observation is the down-regulation of the $[Ca^{2+}]i$ response

induced by LHRH which has not been studied previously in individual ovarian cells. Finally, the intracellular Ca^{2+} sources are clearly involved in LHRH-induced $[Ca^{2+}]i$ changes, whereas the role of extracellular Ca^{2+} needs to be further investigated. These results indicate that LHRH may function as a paracrine or autocrine mediator in the rat ovary with calcium functioning as a second messenger for LHRH.

Chapter 4. LHRH Action on Ovarian Hormone Production: Alterations of Progesterone and Prostaglandins Accumulation by Calcium Ionophore and Protein Kinase C Activator

I. Introduction

Several laboratories have already reported that activation of protein kinase C stimulates basal P_4 production in rat granulosa cells, but inhibits the P_4 response to stimulation by gonadotropins or cAMP derivatives (Shinohara et al., 1986; Kawai and Clark, 1985; Welsh et al., 1984; Leung et al., 1988). The steroidogenic effect of LHRH is partially blocked by a potent inhibitor of PKC (Wang and Leung, 1987). Recently, PKC activity has been characterized in ovarian tissues (Noland and Dimino, 1986; Davis and Clark, 1983; Veldhuis and Demers, 1986). The highest specific enzyme activities are found in the cytosol, followed by microsomes and mitochondria, respectively. In addition, it has been observed that LHRH and its agonists rapidly increase cytosolic free Ca^{2+} level in populations of granulosa cells as measured by quin 2 (Davis et al., 1986), and in individual granulosa cells by fura-2 fluorescence (chapter 2). Although the addition of the calcium ionophore A23187 by itself slightly enhances basal P_4 production in granulosa cells, the calcium ionophore markedly antagonizes the stimulation of P_4 by gonadotropins or CT or cAMP derivatives (Leung et al., 1988). Further, calcium is required in the inhibitory and stimulatory actions of LHRH on

cAMP and steroid production during long-term incubation of ovarian cells (Ranta et al., 1983; Dorflinger et al., 1984; Eckstein et al., 1986). Thus, at the level of the ovarian cell, the hydrolysis of inositol lipids may immediately follow LHRH receptor occupancy and lead to the rapid generation of IP_3 and DG. The resultant changes in calcium mobilization and/or PKC activity may well be involved in the modulatory effects of LHRH on ovarian hormone synthesis.

The present study was performed to elucidate the mechanism of LHRH action on P_4 and PGs synthesis during the different culture periods, the role of calcium and PKC in the LHRH action, and the interaction between IP_3/Ca^{2+}, DG/PKC and cAMP pathways on ovarian hormone production in rat granulosa cells.

II. Materials and Methods

Preparation of animals and granulosa cells

Animals and granulosa cells were prepared as those described in the Chapter 2.

Hormone and drug preparation

Granulosa cells were treated with various hormones and drugs. Melittin, CT, LHRH and FSH were dissolved in saline. AA was dissolved in ethanol. 12-O-tetradecanoylphorbol-13-acetate (TPA) was dissolved in dimethylsulfoxide (DMSO). All drugs were diluted to their respective working concentrations

with MEM before use and added in 5 µl aliquots to a total incubation volume of 1 ml. Control incubations received the same volume of ethanol and DMSO. The final concentration of ethanol or DMSO in the incubations did not exceed 0.5%. At the end of a 5h incubation period, the culture medium was collected and stored at -20°C until assay. Cell viability, as determined by trypan blue exclusion, was not affected by the various treatments.

Progesterone assay

The P_4 concentration in the culture medium was determined by a specific RIA with an antiserum kindly provided by Dr. D.T. Armstrong of the University of Western Ontario (Leung and Armstrong, 1978). The intra-assay coefficient of variation was 5.0%, and coefficient of inter-assay variation was 5.9% (n=25).

Prostaglandin assay

The PGE_2 and PGF_{2alpha} concentrations in the culture medium were determined by RIA with an antiserum kindly provided by Dr. T.G. Kennedy of the University of Western Ontario. The RIA procedure was similar to that described previously (Kennedy, 1979), except that aliquots of the culture medium were assayed without extraction (Hirst et al., 1988). The intra-assay coefficient of variation of PGE_2 was 6.7% and coefficient of inter-assay variation was 9.6% (n=20). The coefficient of intra-assay and inter-assay variation for the PGF_{2alpha} assay were 6.8% and 5.7% (n=5), respectively.

Reagents

The following drugs and hormones were from Sigma: AA, A23187, melittin, TPA, and LHRH. Ovine FSH (NIH-oFSH-16) and pregnant mare's serum gonadotropin were gifts from the NIDDK and the National Hormone and Pituitary Program (University of Maryland School of Medicine). Penicillin-streptomycin, L-glutamine, nonessential amino acids, trypan blue were obtained from Gibco. [1,2-^{3}H(N)]Progesterone (specific activity 115.0 Ci/mmol), [5,6,8,11,12,14,15,-^{3}H(N)]Prostaglandin-F_{2alpha} (specific activity 100-200 Ci/mmol) and [5,6,8,11,14,15-^{3}H(N)] Prostaglandin-E_2 (specific activity 100-200 Ci/mmol) were purchased from New England Nuclear Inc.

Statistical analysis

Statistical significance among groups was calculated by analysis of variance followed by Scheffe's multiple range test. All results were represented as the mean ± SE of determinations of quadruplicate cell cultures of individual treatments in each experiment. In all cases, identical or similar results were observed in at least two or more independent experiments. $P<0.05$ was considered significant.

III. Results

Effects of melittin, LHRH and TPA on progesterone and PGE_2 production

To determine how LHRH and TPA stimulate ovarian hormone production, especially PGE_2 formation, a phospholipase A_2 stimulator, melittin, was added to the medium of granulosa cell culture to increase intracellular free AA.

As shown in the upper panel of Fig. 29, melittin ($3x10^{-7}M$), LHRH ($10^{-6}M$) and TPA ($10^{-7}M$) alone stimulated P_4 accumulation 2 fold, 4 fold and 4.1 fold, respectively, during a 5h granulosa cell culture ($P<0.01$). Concomitant treatment of granulosa cells with melittin with LHRH did not further increase P_4 production. To examine if endogenous AA could synergize with protein kinase C, melittin was added with TPA to granulosa cell. Again, melittin and TPA failed to further enhance the accumulation of P_4 when compared with TPA alone.

As shown in the lower panel of Fig. 29, the PGE_2 concentrations in the culture medium was also determined in the same experiments. Melittin induced a 2.6 fold increase in PGE_2 compared with control (51.9 pg/ml). LHRH caused a 3.2 fold increase in PGE_2 production and TPA also increased PGE_2 production 1.9 fold when compared with control. Interestingly, concomitant presence of melittin with LHRH or with TPA further enhanced the production of PGE_2 ($P<0.01$), which was different from the effects of melittin with LHRH or TPA on P_4 production.

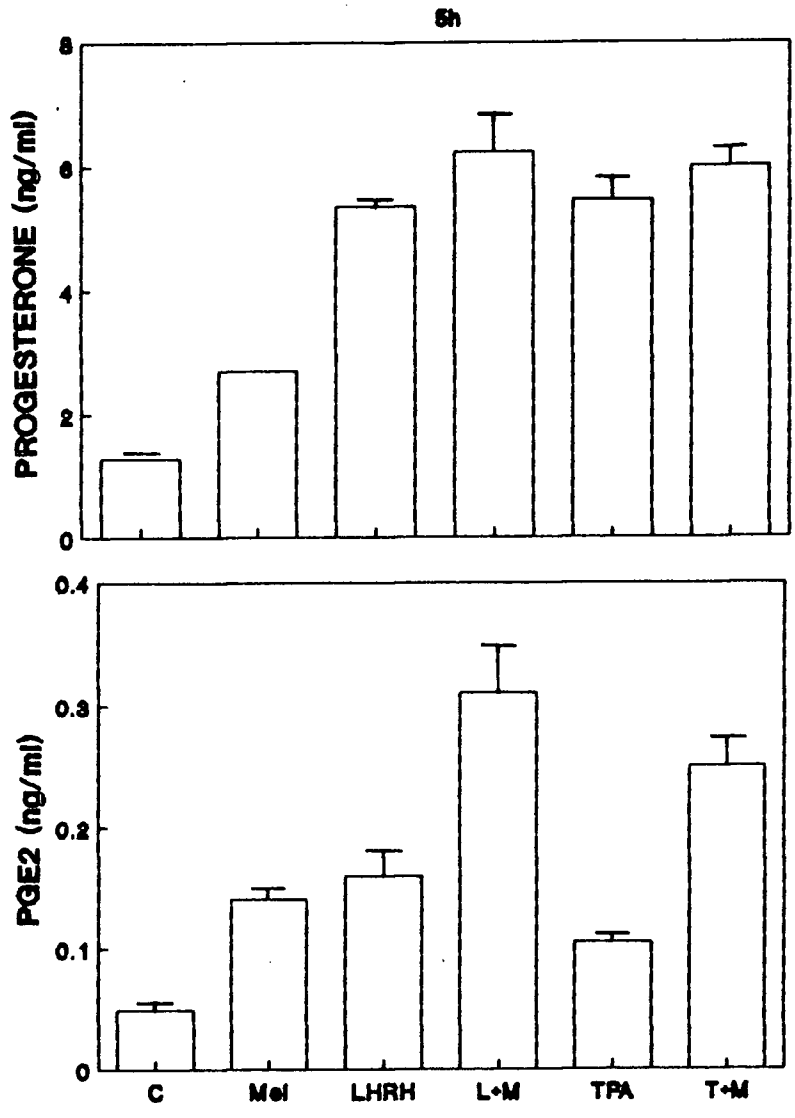

Fig. 29. Interaction of melittin (Mel, M; $3x10^{-7}$M), with LHRH (L; 10^{-6}M) or the phorbol ester TPA (T; 10^{-7}M) on progesterone (PROG) production (upper panel) and PGE_2 formation (lower panel) during a 5h culture. Concomitant presence of melittin with LHRH or with TPA further enhanced the production of PGE_2, while melittin plus LHRH or TPA had no synergistic effect on progesterone production.

Effects of melittin and the calcium ionophore A23187 on progesterone and PGE_2 production

To further investigate the intracellular mechanisms regulating P_4 and PGE_2 formation, granulosa cells were treated with melittin, A23187 and melittin plus A23187 for 5h. As illustrated in the lower panel of Fig. 30, treatment of the cells with melittin ($3x10^{-7}M$) or A23187 ($10^{-7}M$) alone stimulated PGE_2 formation by 1.9 fold and 3 fold, respectively. When both melittin and A23187 were present together, PGE_2 formation was stimulated by 5.2 fold. Interestingly, melittin or A23187 alone also increased P_4 production (upper panel of Fig. 30). However, when both melittin and A23187 were present in the same incubations, P_4 production was not significantly affected when compared with the response to either treatment alone.

Interaction of the calcium ionophore A23187 and TPA: dose response

The possible interaction between calcium and protein kinase C pathways was further examined. The lower panel of Fig. 31 illustrates the synergistic effects of a single dose of TPA ($10^{-7}M$) and increasing concentrations of A23187 ($10^{-9}M$ to $10^{-7}M$). At $10^{-7}M$, the phorbol ester TPA alone stimulated P_4 and PGE_2 production. There was no further enhancement of P_4 accumulation when both TPA and A23187 were present together as compared with the effect of TPA by itself. In contrast, the presence of TPA significantly augmented the stimulation of PGE_2

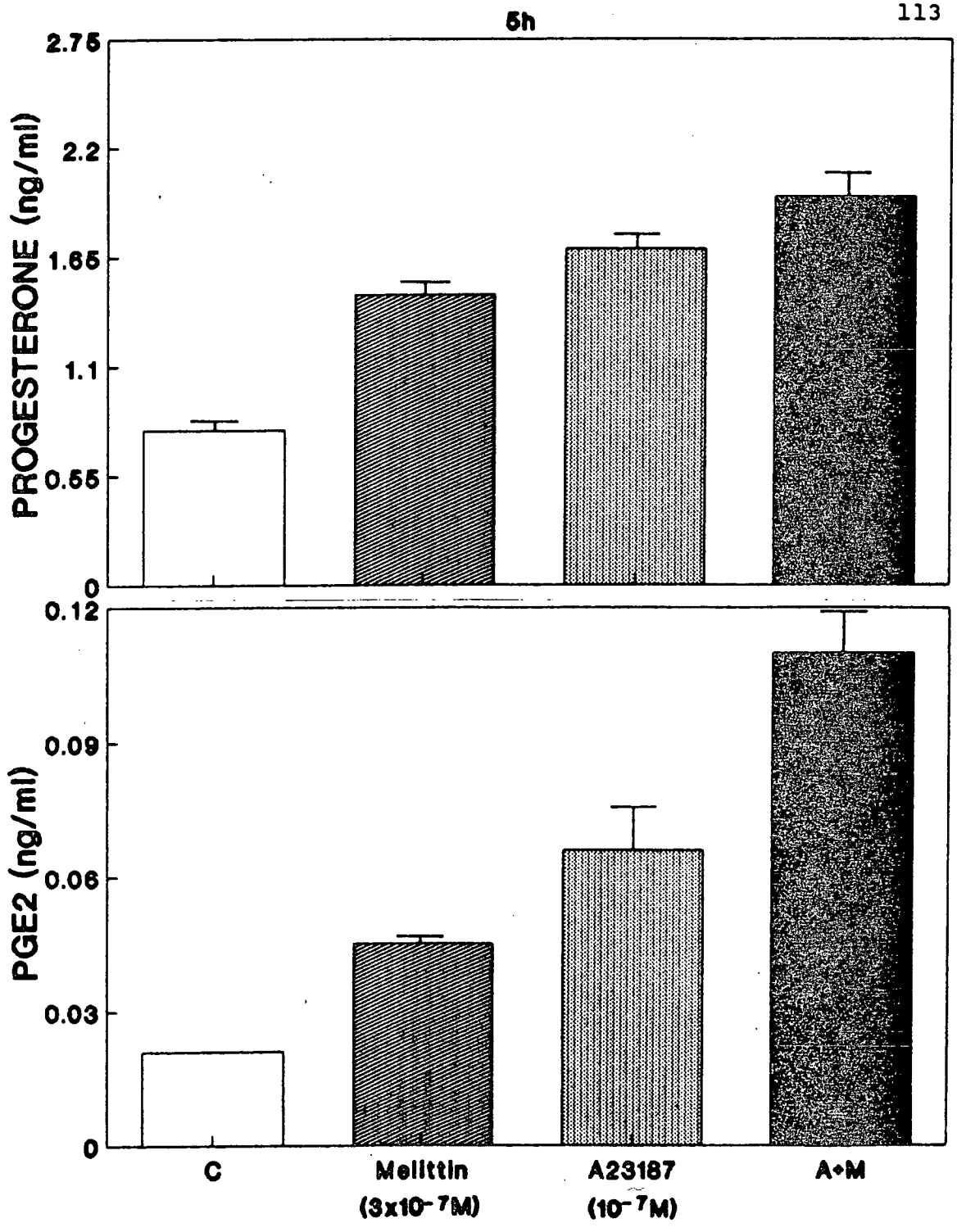

Fig. 30. Effects of melittin and/or A23187 on progesterone and PGE_2 production during a 5h culture period. "A+M" denotes cells treated with both A23187 and melittin. Treatment of the cells with melittin or A23187 alone stimulated both progesterone and PGE_2. When both melittin and A23187 were present, PGE_2 formation was further increased.

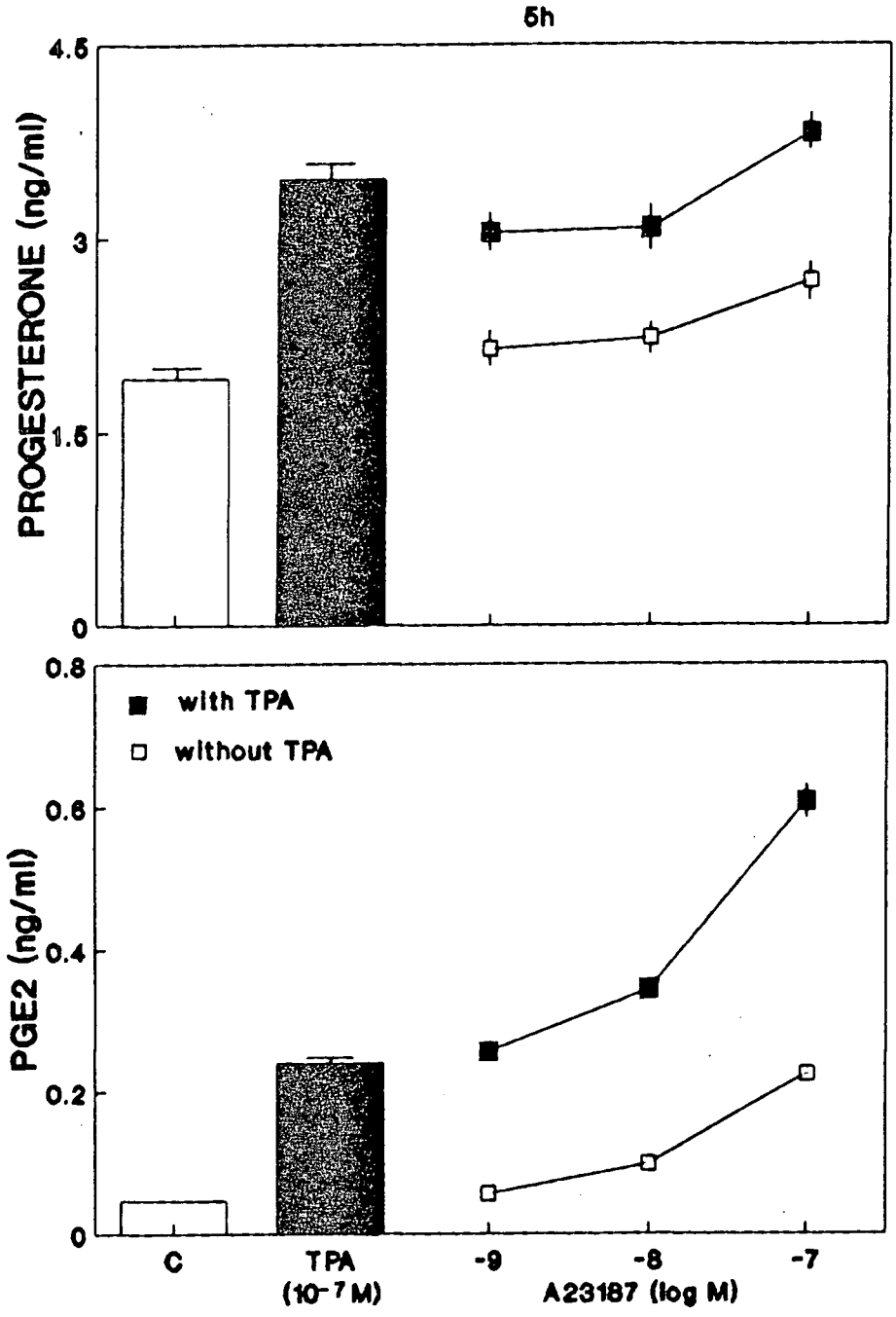

Fig. 31. Effects of the phorbol ester TPA and/or increasing concentrations of the calcium ionophore A23187 on progesterone and PGE_2 production during a 5h culture period. A dose dependent increase in both progesterone and PGE_2 was observed with A23187 treatment. The presence of TPA markedly potentiated the stimulatory action of A23187 on PGE_2 production (lower panel) but not on progesterone (upper panel).

production by A23187.

Fig. 32 shows the effects of A23187 (10^{-8}M) and increasing concentrations of TPA (10^{-10}M to 10^{-8}M) on P_4 and PGE_2 production. A23187 at 10^{-8}M did not affect P_4 production (upper panel of Fig. 32) but significantly increased PGE_2 accumulation. A dose dependent increase in both P_4 and PGE_2 was observed with TPA treatment. When A23187 and TPA were present together in the culture medium, there was no significant alteration in P_4 levels when compared with TPA or A23187 treatment alone. In contrast, the presence of A23187 markedly potentiated the stimulatory action of the different doses of TPA on PGE_2 production (lower panel of Fig. 32).

Interaction of the calcium ionophore A23187, TPA and melittin on PGE_2 production

Based on the above observations, the interaction between melittin, TPA and/or A23187 on PGE_2 production was further examined. As expected, treatment of granulosa cells with TPA (10^{-8}M) or A23187 (10^{-7}M) alone resulted in a significant increase of PGE_2 formation (Fig. 33). Melittin at 3×10^{-7}M increased PGE_2 formation on its own, and further enhanced PGE_2 formation induced by TPA or A23187. Interestingly, while the combined treatment of the cells with TPA plus A23187 exerted synergistic stimulation of PGE_2 formation, the addition of melittin to these cells did not further increase the high levels of PGE_2 accumulation.

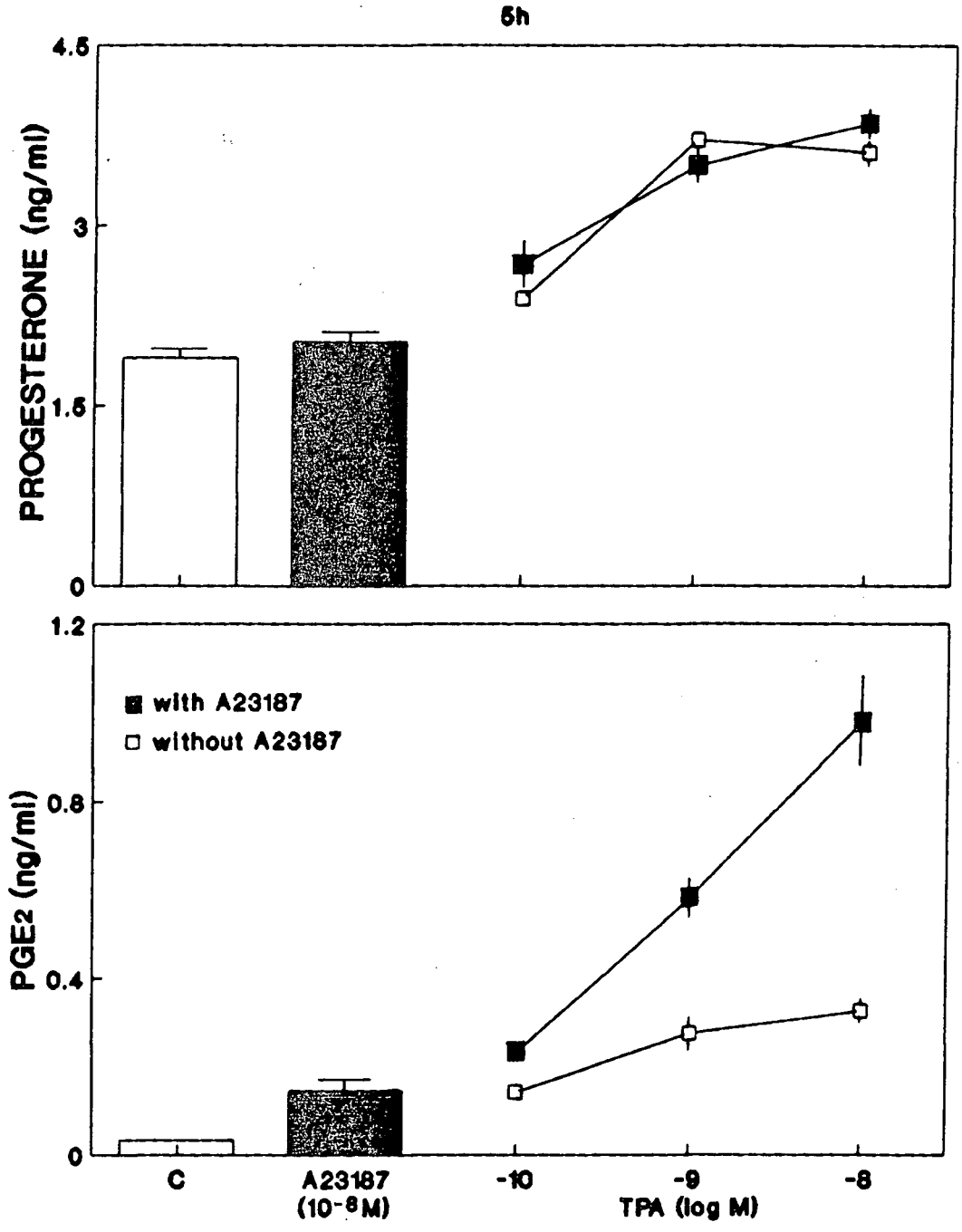

Fig. 32. Effects of the calcium ionophore A23187 and/or increasing concentrations of the phorbol ester TPA on progesterone and PGE_2 production. A23187 alone did not affect progesteron production (upper panel) but increased PGE_2 production (lower panel). The presence of A23187 markedly potentiated the dose-dependent stimulatory action of TPA on PGE_2 (lower panel).

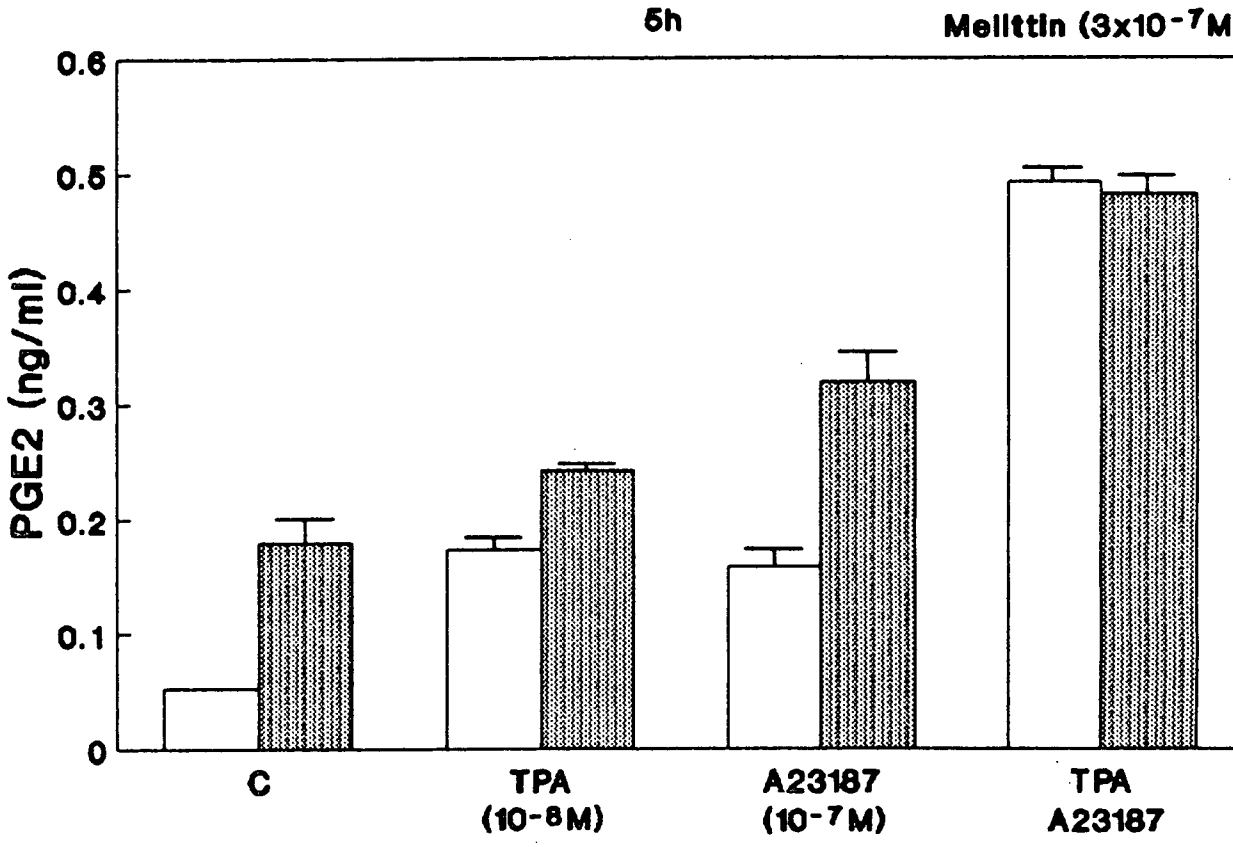

Fig. 33. Effects of the calcium ionophore A23187 and/or the phorbol ester TPA on PGE_2 production, either in the absence (open bars) or presence (hatched bars) of melittin during a 5h culture. The addition of melittin to the cells did not further increase the PGE_2 production induced by A23187 plus TPA.

Effects of cholera toxin and LHRH on progesterone and PGE_2 production during a 5h culture period

Granulosa cells were incubated in the presence of CT (100 ng/ml), LHRH (10^{-6}M) or with CT plus LHRH for 5h. As expected, CT induced a marked increase (14 fold) in P_4 formation, while LHRH caused a relatively smaller (3.5 fold) elevation in P_4 production when compared with control levels (Fig. 34, upper panel). There was no significant difference between the P_4 levels induced by CT alone or by CT plus LHRH.

In addition to P_4, the accumulation of PGE_2 was also determined in the same experiments (Fig. 34, lower panel). Addition of either CT or LHRH stimulated PGE_2 formation, by 3.8 fold and 5.5 fold, respectively. An additive effect on PGE_2 production was observed when both CT and LHRH were present during the incubation.

Effects of the calcium ionophore A23187 and/or cholera toxin on P_4 and PGE_2 production during a 5h incubation period

A calcium ionophore, A23187 (10^{-7}M), was used to mimic the action of LHRH on hormone production. As illustrated in Fig. 35 (upper panel), CT caused a 11 fold increase of P_4 accumulation, whereas only a 94% increase ($P<0.05$) of P_4 level was observed with the A23187 treatment. Combined treatment of granulosa cells with CT plus A23187 significantly attenuated P_4 accumulation compared with the effect of CT alone.

The lower panel of Fig. 35 shows the effect of CT and/or A23187 on PGE_2 formation. In the same incubations, treatment

with either CT or A23187 alone significantly stimulated PGE_2 formation, by 6.5 fold and 3.1 fold, respectively. Combined treatment of the cells with CT plus A23187 induced a further increase in PGE_2 levels, to 10.9 fold when compared with control levels at the end of 5h culture period.

Interaction of the calcium ionophore A23187, TPA and FSH on progesterone and PGE_2 production during a 5h culture period

To further investigate the interaction of calcium, protein kinase C and cAMP pathways on P_4 and PGE_2 synthesis, granulosa cells were treated with A23187 (10^{-7}M), TPA (10^{-7}M) and FSH (100ng) alone or with different combinations. FSH treatment of the cells caused a 17 fold increase of P_4 production (Fig. 36, upper panel). Concomitant presence of FSH with A23187 or TPA resulted in the decrease of P_4 production, 36% and 20% ($P<0.05$), respectively. There was no synergistic inhibitory actions on P_4 production with the combined FSH plus A23187 and TPA treatment of the cells. Moreover, the synthesis of PGE_2 in the same experiment was measured (lower panel of Fig. 36), A23187 or TPA could significantly enhance FSH-induced PGE_2 formation. Most interestingly, the presence of A23187, TPA and FSH together could even further stimulate PGE_2 accumulation ($P<0.01$).

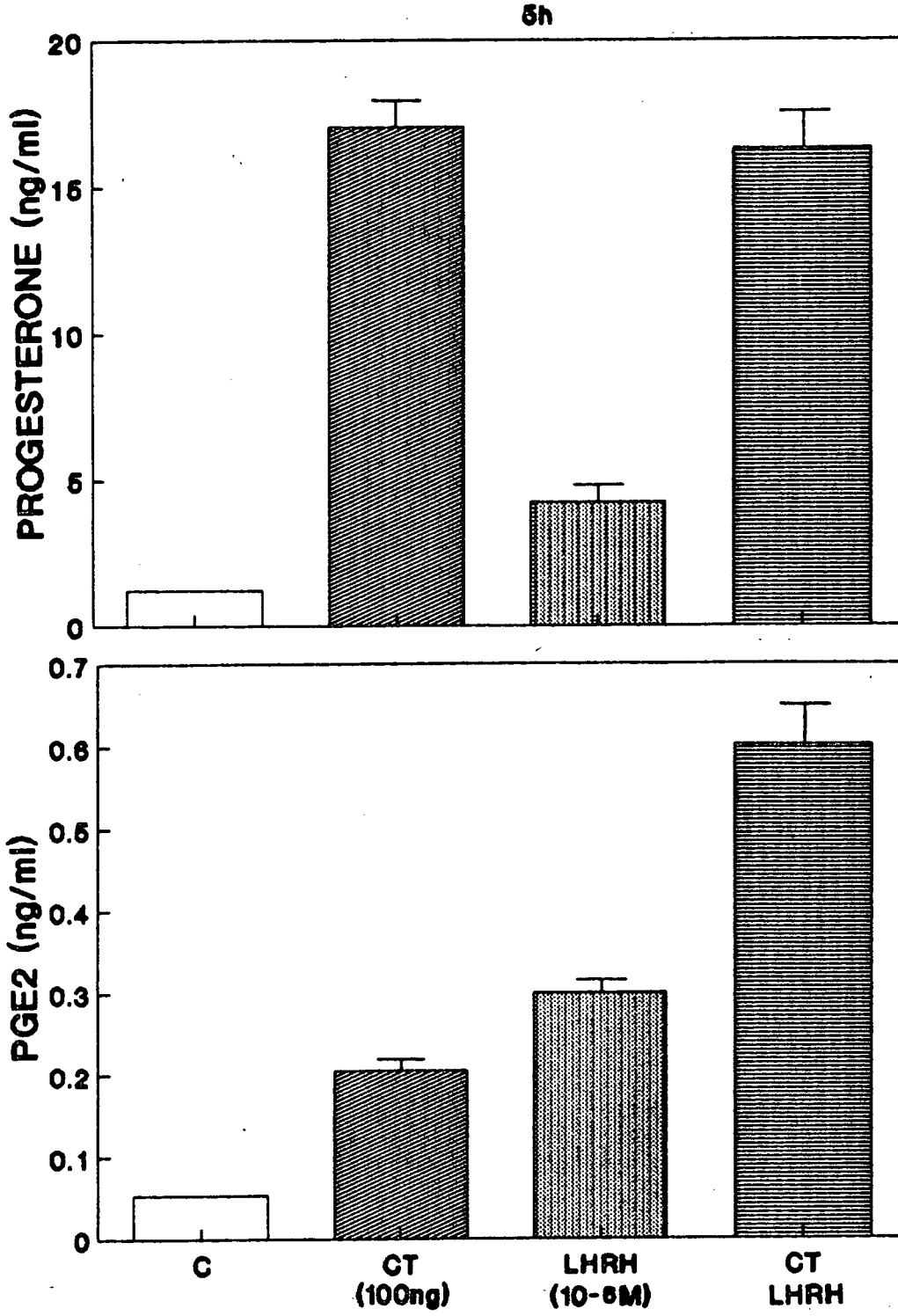

Fig. 34. Effects of cholera toxin (CT) and/or LHRH on progesterone and PGE_2 production during a 5h culture period. Progesterone and PGE_2 production were stimulated by CT or LHRH and an additive effect on PGE_2 production was observed when both CT and LHRH were present.

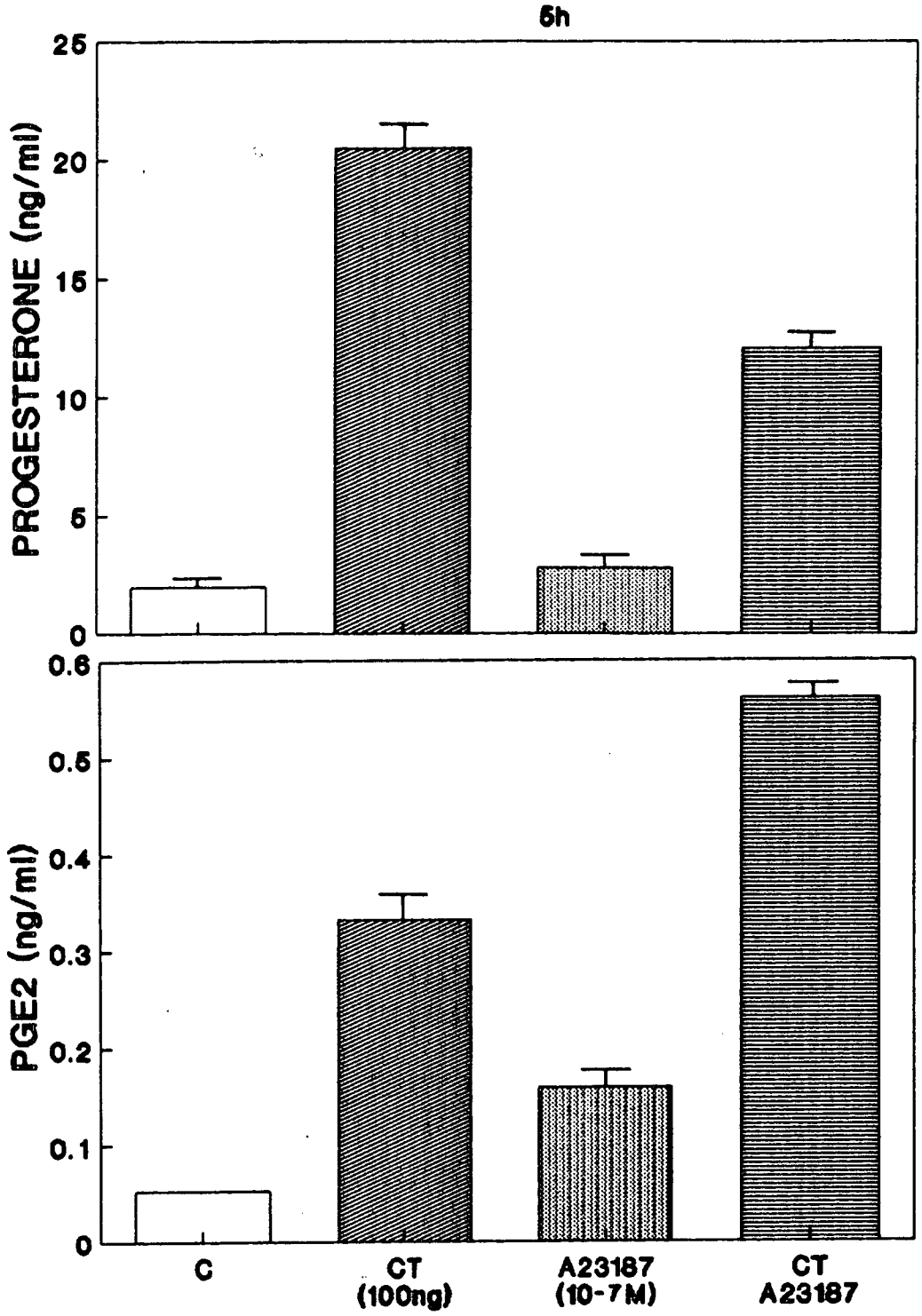

Fig. 35. Effect of the calcium ionophore A23187 and/or cholera toxin (CT) on progesterone and PGE_2 production during a 5h incubation period. Combined treatment of granulosa cells with CT plus A23187 induced a further increase in PGE_2 production compared with each treatment alone.

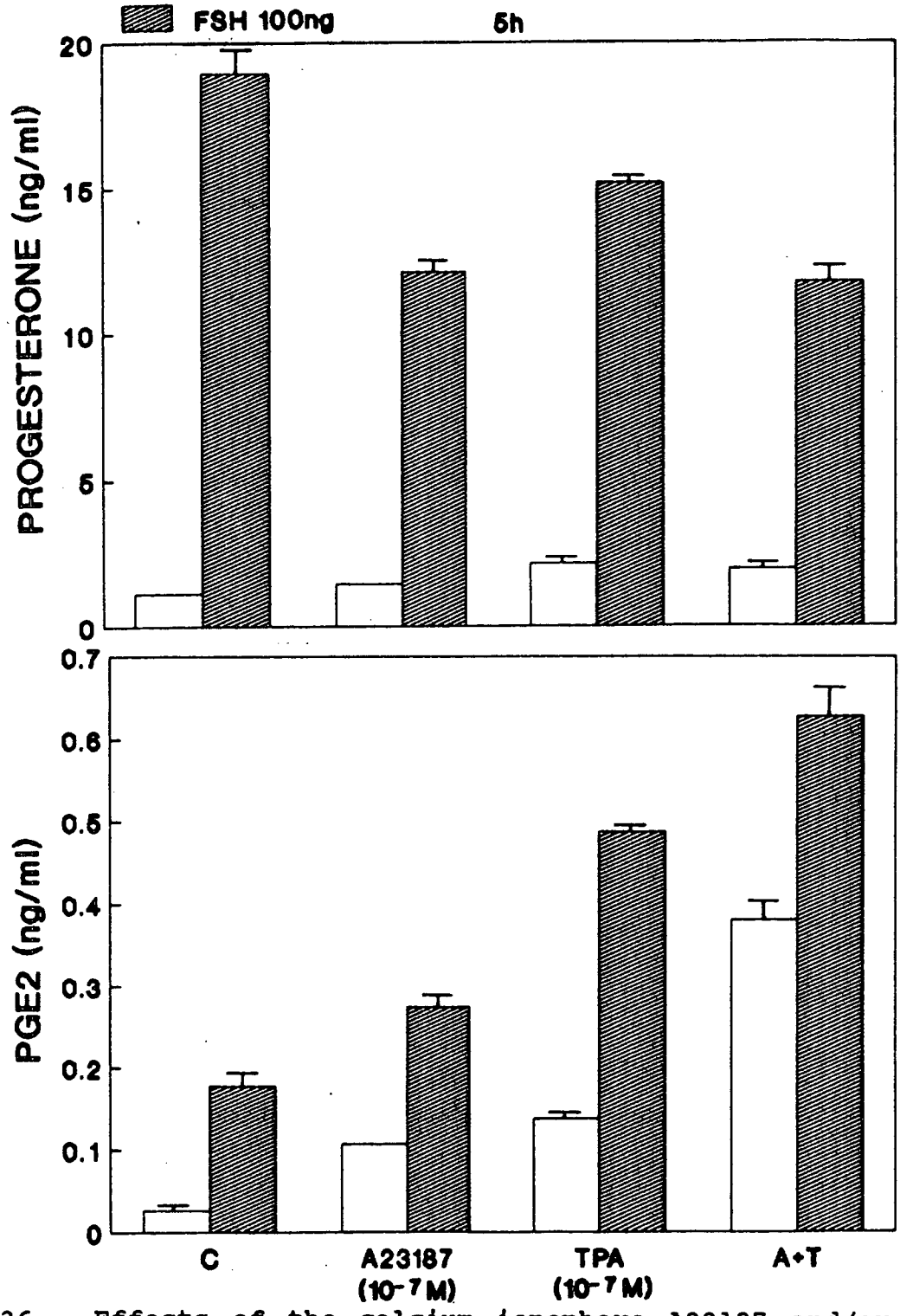

Fig. 36. Effects of the calcium ionophore A23187 and/or the phorbol ester TPA on basal (open bars) or FSH stimulated (hatched bars) progesterone and PGE_2 production during a 5h culture period. "A+T" denotes cells treated with A23187 and TPA concomitantly. A23187 and/or TPA enhanced FSH stimulated PGE_2 production (lower panel), while slightly inhibiting FSH induced P_4 production (upper panel).

Interaction of the calcium ionophore A23187, TPA and cholera toxin on PGE_2 production

The cells were treated with A23187 and/or TPA for 5h, and with or without the concomitant presence of CT (Fig. 37). In the absence of CT, A23187 or TPA by itself stimulated PGE_2 production. Combined treatment of A23187 plus TPA showed synergistic stimulation of PGE_2. CT alone induced a 6.4 fold increase in PGE_2 production. In the presence of CT, TPA or A23187 further augmented PGE_2 formation, by 70% and 25%, respectively, when compared with CT treatment alone. Addition of A23187 plus TPA to the CT-treated cells resulted in the highest increase in PGE_2 formation.

Interaction of FSH and LHRH on the formation of progesterone, PGE_2 and PGF_{2alpha} during a 24h culture period

As shown in the panel A of Fig. 38, FSH significantly stimulated P_4 production during a 24h culture. The production of P_4 induced by FSH was decreased 50% ($P<0.01$) by the concomitant treatment of LHRH (10^{-6}M). LHRH treatment alone slightly stimulated P_4 production but was much less potent than FSH-induced P_4 production. Panel B shows the accumulation of PGE_2 during a 24h culture in the same experiments. Both FSH and LHRH could stimulate PGE_2 production, at least 10 fold, when compared with control; the effect of LHRH was as potent as FSH. Interestingly, addition of LHRH to FSH-treated cells further stimulated PGE_2 formation ($P<0.01$), which was contrary to the effect of LHRH on FSH-induced P_4 production. Panel C of

Fig. 38. shows the effects of LHRH and FSH on the production of PGF_{2alpha}. The basal level of PGF_{2alpha} in control cultures was below the sensitivity of the assay. Treatment with FSH (100ng) or LHRH (10^{-6}M) caused a significant increase of PGF_{2alpha} in the culture medium. Moreover, like their effects on PGE_2, FSH and LHRH synergistically stimulated the production of PGF_{2alpha}.

Interaction of FSH and TPA on progesterone and PGE_2 formation during a 24h culture period.

As the effects of LHRH can be mimicked by the phorbol ester TPA, the action of TPA on FSH-induced P_4 and PGE_2 production was examined. Treatment of rat granulosa cells with TPA, like LHRH, was shown to inhibit FSH-induced P_4 production. The inhibitory effect of TPA was dose dependent, and 10^{-10}M TPA caused a 50% inhibition of P_4 production during a 24h cell culture as shown in the upper panel of Fig. 39. Unlike the inhibitory effect of TPA on FSH-induced progesterone accumulation, TPA resulted in a dose dependent enhancement of PGE_2 production by FSH (Fig. 39, lower panel). The lowest effective dose of TPA was at 10^{-10}M ($P<0.01$).

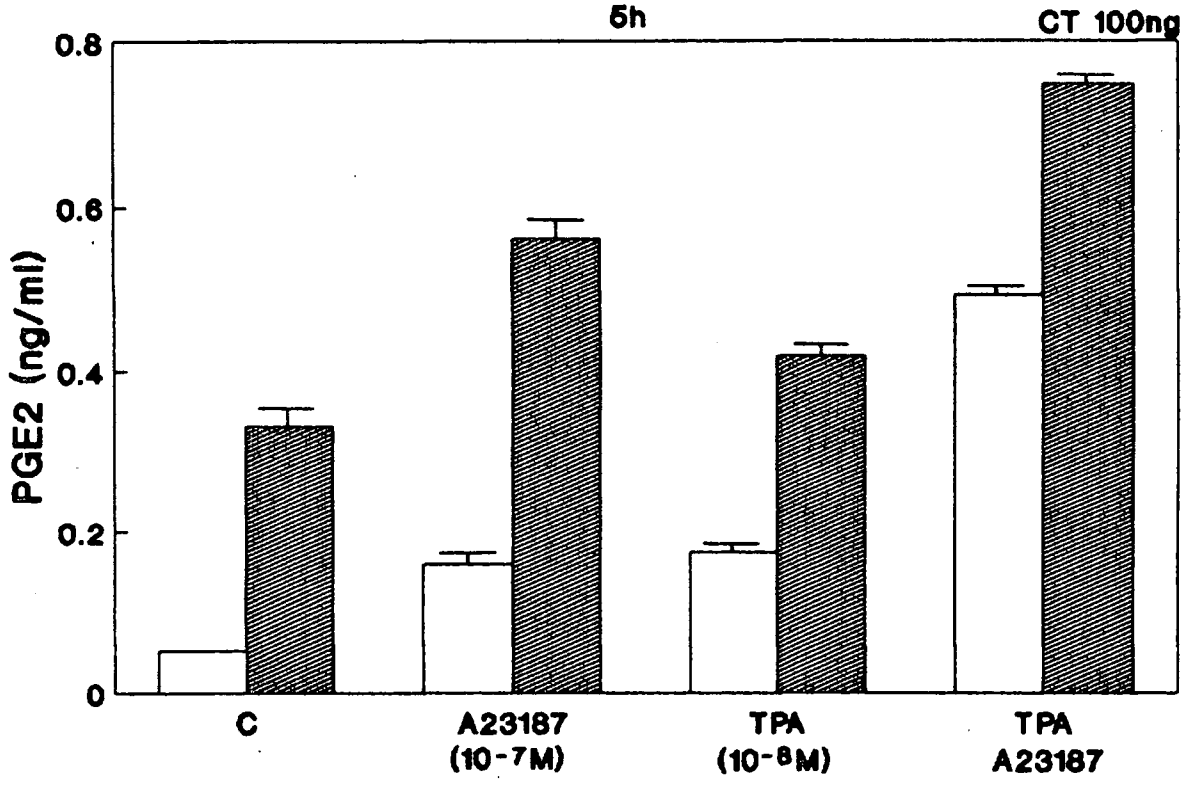

Fig. 37. Effects of the calcium ionophore A23187 and/or the phorbol ester TPA on basal (open bars) or CT stimulated (hatched bars) PGE_2 production during a 5h culture period. CT, A23187 and TPA by itself stimulated PGE_2 production. Addition of A23187 plus TPA to the CT treated cells resulted in the highest increase in PGE_2 formation.

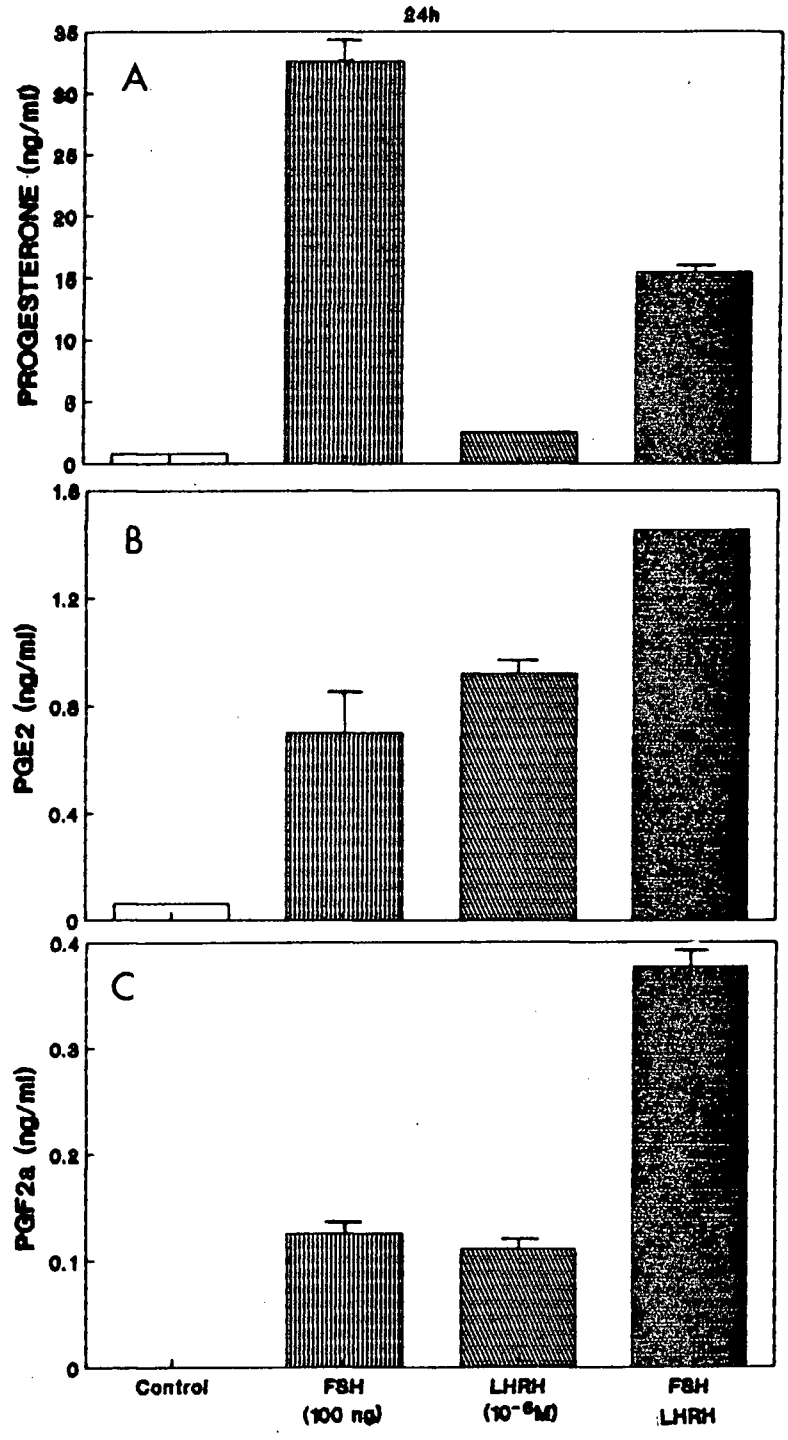

Fig. 38. Interaction of FSH and LHRH on the formation of progesterone (PROG) (panel, A), PGE_2 (panel, B), and PGF_2alpha (panel, C) during a 24h culture period. While LHRH decreased the production of progesterone induced by FSH, LHRH had additive effects on FSH induced PGE_2 and $PGF2_{alpha}$ production.

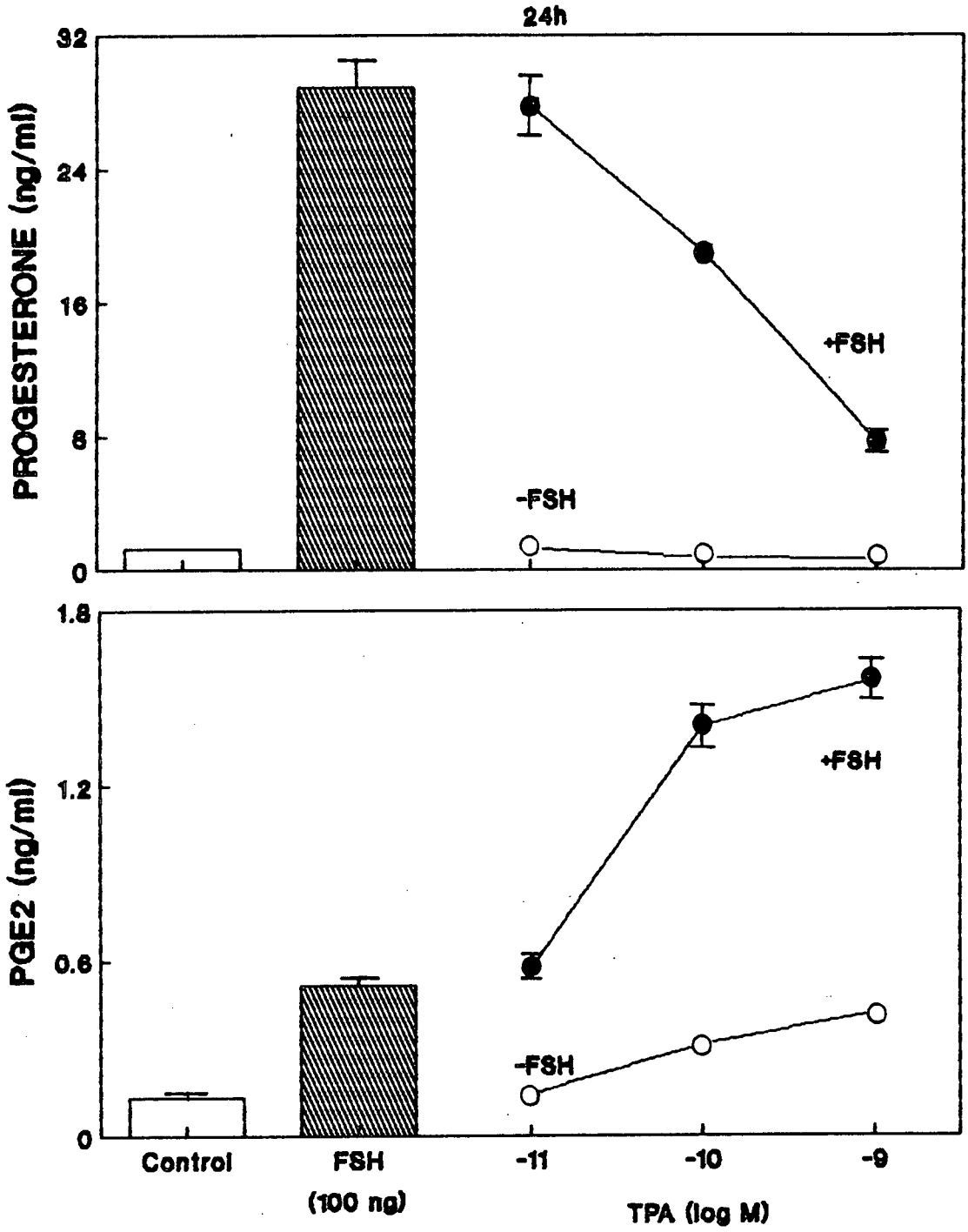

Fig. 39. Interaction of FSH and the phorbol ester TPA on progesterone and PGE_2 formation during a 24h culture period. Treatment of rat granulosa cells with TPA, like LHRH, was shown to inhibit FSH induced progesterone production (upper panel) and enhance PGE_2 production induced by FSH (lower panel).

Interaction of FSH, TPA and the calcium ionophore A23187 on progesterone and PGE_2 formation during a 24h culture period

FSH treatment of granulosa cells caused 18.3 fold increase in P_4 production during a 24h cell culture period (Fig. 40, upper panel). Concomitant presence of A23187 with FSH resulted in a significant decrease in P_4 production, by 40%, when compared with FSH treatment alone. Granulosa cells were also treated with TPA (10^{-11}M to 10^{-9}M). TPA alone at 10^{-9}M slightly decreased the basal level of P_4. With the presence of FSH, TPA induced a concentration dependent decreases in P_4 production but the addition of A23187 (10^{-7}M) did not further potentiate the inhibitory effect of TPA on FSH-induced P_4 production.

In contrast, FSH-stimulated PGE_2 production was further enhanced by the concomitant presence of A23187, from 3.6 fold to 6.4 fold, when compared with the control level of PGE_2. Additionaly, TPA alone caused the concentration dependent increases of basal level of PGE_2. The significant effective dose was at 10^{-10}M. Furthermore, FSH-induced PGE_2 production was enhanced by the concomitant presence TPA and was further augmented by the addition of 10^{-7}M A23187 in the same experiment.

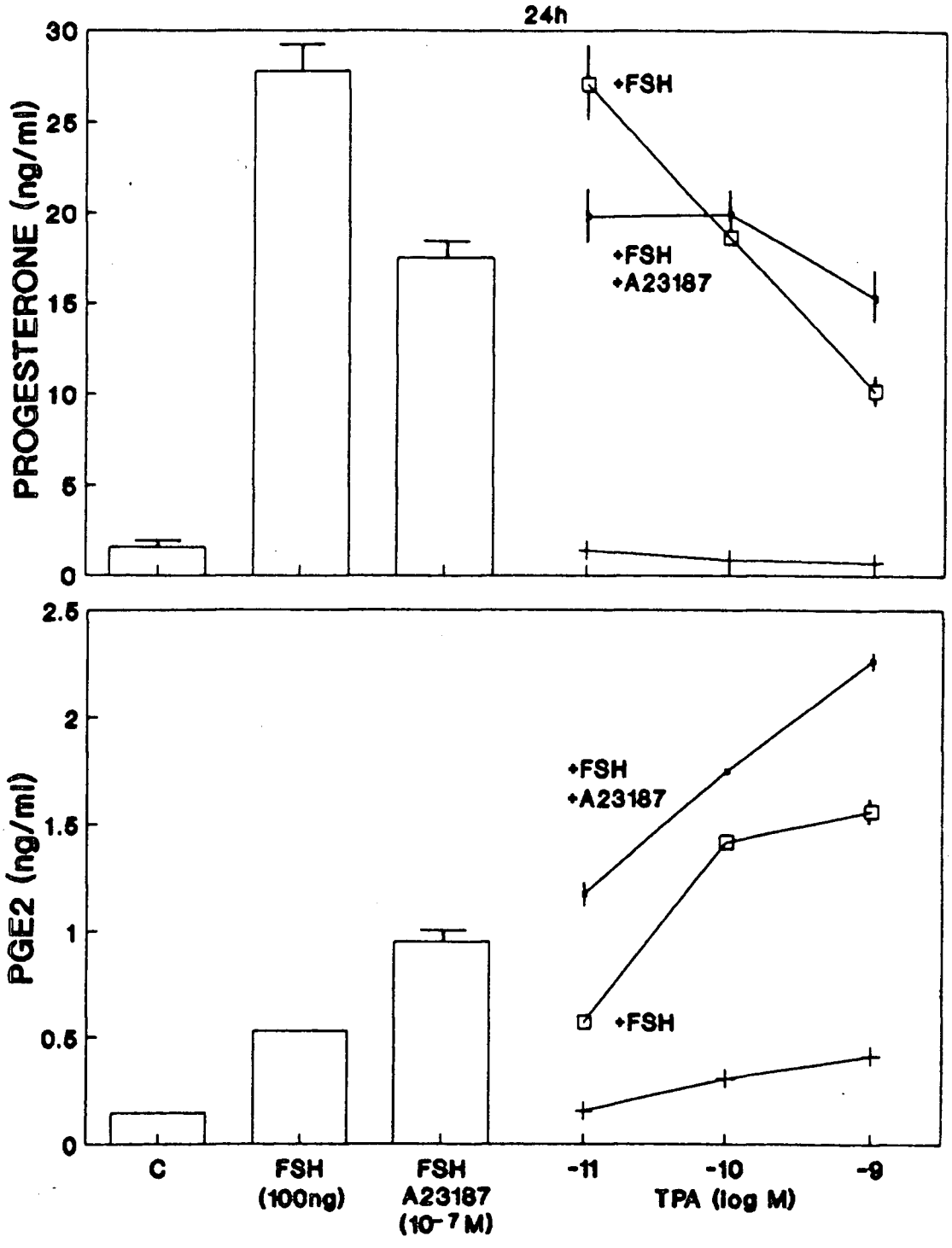

Fig. 40. Interaction of FSH, the phorbol ester TPA and A23187 on progesterone and PGE_2 formation during a 24h culture period. FSH induced progesterone production was inhibited by TPA and/or A23187 (upper panel), while FSH induced PGE_2 production was enhanced by TPA and/or A23187 (lower panel).

IV. Discussion

In addition to its well known stimulatory action on pituitary gonadotrophes, LHRH can exert differential effects on hormonogenesis in the ovary (Hsueh and Jones 1981; Clark, 1984). While the mechanism of action of LHRH at the level of the ovarian cell is not completely understood, there is increasing evidence that inositol lipid may play a role in its effect on PKC and $[Ca^{2+}]i$. Therefore, the hydrolysis of phosphoinositides may immediately follow LHRH receptor occupancy and lead to the rapid generation of diacylglycerol and inositol phosphates (Ma and Leung, 1985; Davis et al., 1986; Leung et al., 1986). The resultant changes in PKC activity, and $[Ca^{2+}]i$ level may well be correlated with the modulatory effects of LHRH on P_4 production. The activation of PKC by TPA has been shown to inhibit FSH-stimulated estrogen, P_4 and 20-alpha-OH-P production. This inhibitory action induced by TPA appears to be specific, as the phorbol congener 4-alpha-phorbol-12,13-didecanoate is ineffective on steroid hormone production (Welsh et al., 1984). TPA and two synthetic diacylglycerols, sn-1,2-dioctanoyl glycerol and 1-oleoyl-2-acetoyl-sn-3-glycerol have been shown to inhibit cAMP dependent granulosa cell differentiation (Shinohara et al., 1985). Structural similarities between DG and TPA suggest that these compounds may stimulate the activation of PKC in the same manner (Hsueh, 1979), and endogenous DG formation induced by LHRH may have similar action as exogenous DG or TPA. In contrast to their inhibitory effect, TPA and DG stimulate the

production of P_4 and PGs without the presence of gonadotropins (Shinohara et al., 1985; Kawai and Clark, 1985; Wang and Leung, 1987). The stimulatory effects of LHRH on P_4 and PGE_2 production in the present study confirmed those of earlier studies (Fig. 29, 31 and 32). In addition, the proposed involvement of PKC activation in mediating the production of P_4 as well as the proposed specificity of the phorbol ester for activation of PKC have been further supported by the inhibitory effect of the PKC inhibitor H-7 on LHRH- or TPA-induced P_4 production (Wang and Leung, 1987). Similarly, the stimulatory effect of TPA on the plasminogen activator activity of hen granulosa cell is also blocked by H-7 (Tilly and Johnson, 1988). Although H-7 may inhibit adenylate cyclase as well as PKC, the stimulatory effect of exogenous cAMP on H-7 treated granulosa cells indicate the intact responsiveness of the cells at a post-cAMP step (Wang and Leung, 1987).

Concerning the role of AA in LHRH action, it has been shown that LHRH could induce AA liberation from membrane phospholipids in [^{3}H] AA prelabeled granulosa cells (Minegishi and Leung, 1985; Kawai and clark, 1986). Thus, LHRH stimulated PGE_2 increase could be related to the accumulation of intracellular free AA which serves as the substrate for PGs and leukotrienes (LTs) formation. The effect of AA may be due to one or more of its metabolites (Hirst et al., 1988). Although AA is extensively present in the body, practically all of it is esterified on phospho or neutral lipid (Irvine, 1982). Therefore the hydrolysis of esterified AA provides the first

rate-controlling step in PGs and LTs formation by limiting the amount of substrate available to cyclooxygenase and lipoxygenase.

Melittin, a polypeptide from the venom of honey bee which can activate PLA_2, has been used to enhance intracellular free arachidonic acid concentrations (Haberman, 1972). In the present study, the effect of melittin on P_4 production was measured after 5h incubation, and melittin induced a 2 fold increase in P_4 (Fig. 29). This result suggests that endogenous AA release may also be involved in the regulation of P_4 production. To further examine if the endogenous AA release interacts with PKC pathway on ovarian hormone production, melittin and LHRH were added concomitantly to the cell culture, and they did not further increase P_4 production (Fig. 29, upper panel). It appears that the effect of LHRH on P_4 production already includes the action of endogenous AA. AA induced by LHRH could be liberated from membrane phospholipids by two different mechanisms to form its metabolites. AA can be hydrolyzed from phospholipids by a Ca^{+2} dependent process involving phospholipase A_2, or alternatively from inositol lipids through two consecutive reactions catalyzed by phospholipase C and DG lipase (Lapetina et al., 1981; Bell and Majerus, 1980). If LHRH stimulated PLA_2 and PKC separately, the combined treatment of melittin and TPA should enhance the accumulation of progesterone to a greater extent than each agent alone (Fig. 29, upper panel). However the formation of P_4 remained at the same level in the presence of LHRH plus TPA

as that caused by TPA alone. This suggests that the liberation of AA by LHRH could be distal to the activation of PKC. In addition, the previous observations of the stimulatory effects of melittin on LH and ACTH release further support the concept that the activation of PLA_2 could participate in the action of hormones (Chang et al., 1986; Aboutsamra et al., 1986).

LHRH not only stimulated basal P_4 production, but also enhanced the formation of PGE_2 (Fig. 29, lower panel). In the present study, LHRH and TPA were found to act synergistically with melittin on the accumulation of PGE_2. Since the effect of melittin on the formation of PGE_2 is probably due to its ability to increase intracellular unesterified AA, the present results indicate that LHRH could further regulate the activity of cyclooxygenase to elicit the formation of PGE_2 when the unesterified AA substrate is increased by melittin. TPA could mimic the effect of LHRH, indicating involvement of a PKC dependent mechanism. On the other hand, it has been observed that TPA potentiates A23187 induced AA release (chapter 2). These results indicate that LHRH-induced activation of PKC is involved in a two-step process of PGE_2 production: first AA is released from membrane phospholipids and second cyclooxygenase is activated.

In the present study, the granulosa cells were cultured with different treatments for hours to detect the hormone production, which was much longer than that taken up (seconds or minutes) for the measurements of the inositol phosphate formation and the $[Ca^{2+}]i$ change. Earlier time points have not

been explored because the production of P_4 and PGE_2 were below the sensitivity of RIA. On the other hand, it has become apparent that in many cells in which the calcium messenger system controls sustained cellular response there is no simple correlation between the increase in the $[Ca^{2+}]i$ and the cellular response (Rink et al., 1982; O'Doberty et al., 1980; Charest et al., 1983). In some systems, this phenomenon is called "hysteresis" (Rasmussen, 1983). One explanation is that a larger increase in $[Ca^{2+}]i$ is required to activate Ca^{2+} dependent processes, such as PKC activity, than to control sustained cellular response. It has been suggested that PKC and Ca^{2+} pathway may have distinct functional roles. For example, in pancreatic islets, Ca^{2+} is largely responsible for initiating the cellular response, whereas PKC is largely responsible for maintaining cellular response (Rasmussen et al., 1984). It has also been observed that addition of Ca^{2+} to the cultured pituitary cells in Ca^{2+} free medium causes an increase in PRL production. Increase in PRL synthesis is not observed for several hours, but once synthesized, this increase in PRL may be sustained for days (Gick and Bancroft, 1985). In a similar manner, LHRH induces rapid and transient increase in $[Ca^{2+}]i$ which may then go on to control the sustained ovarian hormone production.

In an attempt to modify intracellular levels of calcium more directly, A23187, a divalent cation ionophore which facilitates the transmembrane transport of calcium, was used in the present study. A23187 alone stimulated both P_4 and PGE_2

production during a 5h culture period (Fig. 30). The stimulatory effect of A23187 on the production of P_4 was probably due to the conversion of pregnenolone to progesterone but not the synthesis of this intermediate from cholesterol as previously proposed (Carnegie and Tsang, 1987). Interestingly, treatment of granulosa cells with melittin plus A23187 further enhanced PGE_2 production (Fig. 30, lower panel). A23187 could also stimulate AA release from rat granulosa cells (Minegishi et al., 1987). These data imply that calcium plays modulatory roles in both the release of AA by the activity of phospholipase A_2 and the conversion of AA to PGE_2 by cyclooxygenase.

In many systems, a dual signal transduction mechanism exists that involves the Ca^{+2} and PKC pathways acting either cooperatively or synergistically to give the maximal response to a given hormonal signal (Nishizuka et al., 1984; Berridge, 1987). Huckle and Conn have observed that both calcium and PKC are involved in the action of LHRH on gonadotropins release from the anterior pituitary (Huckle and Conn, 1987). The synergistic effects on LH release are noted with the calcium ionophore A23187 and PKC activators such as the phorbol ester TPA, possibly through enhanced activation of PKC (Harris et al., 1985). Combination of TPA and A23187 causes larger amounts of sustained release of PRL compared to each compound alone (Delbeke et al., 1984). The present study clearly demonstrated that TPA failed to act synergistically with A23187 on P_4 production, whereas TPA and A23187 were synergistic on

PGE_2 production (Fig. 31-33). While A23187 has been shown to enhance acute TPA-induced production of PGF_{2alpha} in swine granulosa cells (Veldhuis and Demers, 1987), a report on the inhibitory effects of TPA and A23187 on LH induced P_4 production in rat luteal cells has not described this potential synergism (Baum and Rosberg, 1987).

It has been shown that in parotid gland, where PKC and Ca^{2+} synergistically induce enzyme secretion, Ca^{2+} alone is responsible for control of plasma membrane K^+ channels (Putney et al., 1984). In the present study, TPA did not act synergistically with A23187 on P_4 production but did act synergistically with A23187 on PGE_2 formation (Fig. 31 and 32). Taken together, these results suggest the potential for a degree of independent control of the cell responses that are unaffected by the synergism of PKC and Ca^{2+}.

As proposed previously, addition of EDTA to the granulosa cells partially reversed the inhibitory effect of LHRH and TPA upon FSH-induced P_4 production, while the reversal effect of EDTA could be completely abolished by the simultaneous addition of Ca^{2+} (Leung and Wang, 1988). These results indicate that the action of LHRH on ovarian P_4 steroidogegesis is mediated at least in part by a Ca^{2+} dependent PKC.

The present studies also showed that the formation of P_4 was not coupled tightly to the production of PGE_2. The treatments of granulosa cells with melittin plus A23187, TPA plus melittin and TPA plus A23187 only increased PGE_2 formation but not P_4 production to higher amounts (Fig. 31 and 32).

Furthermore, in the presence of both TPA and A23187, the formation of PGE_2 was not further augmented by melittin. This perhaps reflects the already maximal conversion of AA to PGE_2 under the influence of TPA plus A23187 (Fig. 33).

The interactions between Ca^{2+}-PKC and cAMP pathways were also investigated in the present study. Treatment of rat granulosa cells with LHRH further enhanced CT-stimulated PGE_2 production but failed to affect the production of P_4 induced by CT during a 5h incubation period (Fig. 34). This result supports the previous observation that the mechanism of LHRH on PGs production is distinguishable from that of gonadotropins (Clark, 1982). While LH increases PG production, it does not increase [^{3}H]AA release (see Chapter 2). Therefore, the effect of LH on PG production in granulosa cells is presumably at a step in the PG synthesis after hydrolysis of AA, and may involve an increase in PG synthetase activity (Clark et al., 1980; Koos and Clark, 1982). A23187 and TPA were used to mimic the action of LHRH on PGE_2 formation with the presence of FSH and CT in the present study. Interestingly, TPA suppressed FSH-induced P_4 production but enhanced PGE_2 formation induced by FSH or CT during a 5h culture (Fig. 36 and 37). Likewise, the role of calcium in ovarian hormone production appears to be complex and may be affected by the presence of gonadotropins or cAMP induced agents. While basal P_4 formation was increased only marginally by 10^{-7}M A23187, PGE_2 production was stimulated by 4.5 fold in the same incubations (Fig. 35 and 36). Moreover, in the presence of FSH or CT, the production of P_4

was attenuated by the concomitant presence of A23187, whereas PGE_2 formation was further augmented (Fig. 35 and 36).

In long term culture (24 h to 48 h), LHRH has been reported to reduce FSH-induced P_4 production (Hsueh and Jones, 1981). In contrast, LHRH acted synergistically with FSH on the formation of PGE_2 and PGF_{2alpha} in the present study (Fig. 38). Since the increase in PGE_2 concentration (panel B of Fig. 38) was about four times greater than that of PGF_{2alpha} (panel C of Fig. 38), only the formation of PGE_2 was determined in the subsequent experiments. Very similar effects were observed with the PKC activator TPA on FSH-induced P_4 and PGE_2 formation (Fig. 39). A23187 potentiated FSH-induced PGE_2 production, and further enhanced PGE_2 formation induced by TPA plus FSH (Fig. 40, lower panel), whereas A23187 inhibited FSH-induced P_4 production (Fig. 40, upper panel). The present short and long term studies clearly strengthen the multiple roles of protein kinase C and Ca^{2+} on hormone production, either stimulatory or inhibitory, depending on the time of culture and the nature of the hormones.

The interaction between cAMP and Ca^{2+}-PKC pathways can occur at different levels. The first is at the level of receptors; signals produced by activation of one pathway may affect the ability of a receptor functioning via the other pathway to generate an intracellular signal in response to an agonist. Secondly, these intracellular messengers may interact in producing the cell response. Finally, these interactions may extend to neighboring cells; thus AA produced after

activation of PLA_2 or PLC followed by DG lipase is the substrate for synthesis of PGs and/or LTs. These metabolites might be released from the cell where they are produced and function as local hormones to mediate the functions of their target cells. Although the inhibitory effect of LHRH and its agonists on P_4 production have been studied extensively, this is the first demonstration of a synergistic effect between LHRH with FSH on ovarian PGE_2 formation, while at the same time inhibiting P_4 accumulation during a 24h culture period (Fig. 38). The inhibitory effect of LHRH, A23187 and TPA on FSH-induced P_4 accumulation may be partialy due to a reduction in cAMP formation (Shinohara et al., 1985; Knecht et al., 1981; Jones and Hsueh, 1981). Previous studies have shown that in cultured theca and granulosa cells, FSH-stimulated PGE production could be mimicked by cAMP in both cell types (Zor et al., 1983; Clark et al., 1978). However, the present observation that LHRH, TPA and A23187 did not antagonize the FSH-induced PGE_2 production, but rather augmented the production of PGs raises the possibility that the stimulatory action of LHRH on PGE_2 production via the activation of PKC and the change of $[Ca^{2+}]i$ is at a post-cAMP step (Fig. 38-40). These data also support the concept that TPA and A23187 inhibit FSH-induced P_4 production, in part at a step or steps beyond cAMP generation and degradation (Leung et al., 1988). Taken together, it can be concluded that cAMP, calcium and protein kinase C pathways are all involved in PGs synthesis, and each pathway is distinguishable from the others in the way that PG

is produced.

Nishizuka (1984) has proposed that two classes of receptors are coupled with cAMP and Ca^{2+}-PKC induced intracellular cascades. In some tissues, the two classes of receptors function in similar direction to cause a full physiological cellular response. In other tissues, the two types of receptor-linked cascades counteract each other, e.g., an extracellular signal that increases cAMP blocks PKC activation, Ca^{2+} mobilization and AA release (Nishizuka, 1984). The present study supports this notion and further indicates that the intracellular process in ovarian cells induced by two major second messenger pathways can either cooperate with or antagonize each other.

The physiological role of LHRH and its interaction with FSH on P_4 and PGE_2 formation is not yet clear at present. A role of PGE_2 to increase plasminogen activation has been proposed (Strickland and Beers, 1976). Furthermore, prostaglandins may be important in follicle growth, oocyte maturation and ovulation (Clark et al., 1978; Naor et al., 1984). On the other hand, it is known that the maturation of rat oocytes could be blocked by cAMP (Dekel and Beers, 1978). LHRH could decrease the formation of cAMP induced by gonadotropins and result in the maturation of oocytes (Hillensjo and Lemaire, 1980; Dekel et al., 1983; Dekel and Aberdam, 1985). The present studies further strengthen the concept that LHRH does not exert an overall inhibitory action on ovary but rather causes specific stimulation of P_4 and PG

synthesis, suggesting that LHRH may exert multiple effects on the ovarian hormone production and the multiple pathways are intimately involved in the actions of LHRH.

Chapter 5. Role of Arachidonic Acid in Luteinizing Hormone-Releasing Hormone Action.

I. Introduction

While the influence of LHRH on ovarian hormone production has been well documented, its mechanism of action at the post-receptor level is still largely unresolved. In the past few years, LHRH and its agonists have been shown to stimulate the breakdown of polyphosphoinositides into inositol phosphates and 1,2 diacylglycerol in the ovary. Phospholipid turnover was usually accompanied by the mobilization of $[Ca^{2+}]i$, and may cause the activation of Ca^{2+} dependent PLA_2, leading to the liberation of AA.

The above hypothesis notwithstanding, it has been proposed that the action of LHRH in the stimulation of LH release from gonadotrophs also involves the release of AA from membrane phospholipids. One or more of the lipoxygenated metabolites or epoxygenated products of AA might be a component of the cascade of reactions initiated by LHRH that ultimately result in the secretion of LH (Naor and Catt, 1981; Snyder et al., 1983). To ascertain whether similar signal transduction mechanisms are evoked after the binding of LHRH to its ovarian receptors, the effect of LHRH on AA release in rat ovarian cells has been recently examined (Minegishi and Leung, 1985). In cultured granulosa cells prelabeled with $[^3H]$AA, LHRH caused a rapid increase in the level of AA release from phospholipids. This increase in AA release was also induced by an agonistic LHRH

analog, but completely blocked by the concomitant presence of a potent LHRH antagonist, suggesting a specific receptor mediated mechanism (Minegishi and Leung, 1985). While these data support the notion that AA liberation from phospholipids is enhanced by LHRH, its relationship to steroid hormone production in the ovary is not understood. Treatment of granulosa cells with melittin resulted in the elevation of basal level of P_4 and PGs (chapter 4), suggesting endogenous AA not only serves as the precursor for PGs synthesis but also exerts its effect on steroidogenesis. The present study was designed to determine if AA could be a mediator of LHRH action during the different culture periods. The involvement of various pathways of AA metabolism in the control of P_4 production was also investigated.

II. Materials and Methods

Animals and granulosa cells preparation

The preparation of animals and granulosa cells were the same as those described in Chapter 2.

Hormone and drug preparation

Granulosa cells were treated with various hormones and drugs. LHRH, FSH, CT and melittin were dissolved in saline. 25-Hydroxycholesterol (25-OH-cholesterol), AA and all hydroperoxy acids were prepared freshly in ethanol. After opening the ampules of AA and hydroperoxy acid, they were

stored under nitrogen at -70°C. TPA, indomethacin, and nordihydroguaiaretic acid (NDGA) were dissolved in DMSO. All drugs were diluted to their respective working concentrations with MEM before use and added in 5 µl aliquots to a total incubation volume of 1 ml. Control incubations received the same volume of ethanol and DMSO. The final concentration of ethanol or DMSO in the incubations did not exceed 0.5%. At the end of different incubation periods, the culture medium was collected and stored at -20°C until assay. For intracellular P_4 production, 0.5 ml of 100% ethanol was added into the culture dishes, and the cells were scraped out with a rubber policeman. The cell suspension was transfered into 10 x 75 mm test tube and mixed vigorously on a vortex. Then the suspension was centrifuged at 3000xg for 10 min at 4°C. Ethanol was poured out into another test tube, dried under nitrogen and redissolved with 200 ul of MEM for RIA.

Progestin assay

The P_4 and 20alpha-hydroxy-4-pregnen-3-one (20alpha-OH-P) concentrations in the culture medium were determined by a specific RIAs with antisera kindly provided by Dr. D.T. Armstrong of the University of Western Ontario. The lowest detectable concentration was 0.08 ng/ml for both P_4 and 20alpha-OH-P. The intra-assay coefficient of variation was 5.0%, and coefficient of inter-assay variation was 5.9% for P_4 assay (n=25). The intra-assay coefficient of variation of 20alpha-OH-P was 7.2% and coefficient of inter-assay variation

was 10% (n=6).

Prostaglandin E_2 assay

The PGE_2 concentrations in the culture medium were determined by RIA with an antiserum kindly provided by Dr. T.G. Kennedy of the university of Western Ontario. The RIA procedure was similar to that described previously (Kennedy, 1979), except that aliquots of the culture medium were assayed without extraction (Hirst et al., 1988). The intra-assay coefficient of variation was 6.7% and coefficient of inter-assay variation was 9.6% (n=20).

Materials

The following were purchased from Sigma (St. Louis, USA): oleic acid (C18:1), 11,14 eicosadenoic acid (C18:2), linoleic acid (C18:2), homo-gamma-linolenic acid (C18:3) (8,11,14 eicosatrienoic acid), gamma-linolenic acid (C18:3), arachidonic acid (C20:4), TPA, LHRH, CT, 25-OH-cholesterol indomethacin, and NDGA. Ovine FSH (NIDDK oFSH-16) and PMSG were gifts from the National Hormone and Pituitary Program NIDDKD, NIH. The LHRH agonist [d-Trp^6,Des-Gly^{10},Pro^9-NHEt]LHRH was kindly supplied by Dr. Nicholas Ling of the Salk Institute (La Jolla, CA). All hydroperoxy fatty acids were purchased from BIOMOL Laboratories (Philadelphia, PA). [1,2-^{3}H(N)]Progesterone (specific activity 115.0 Ci/mmol), 20alpha-[1,2-^{3}H(N)]-hydroxypregn-4-ene-3-one (specific activity 40-60 Ci/mmol) and [5,6,8,11,14,15-^{3}H(N)]Prostaglandin E_2 (specific activity 100-

200 Ci/mmol) were purchased from the New England Nuclear Corporation (Ontario, Canada). Scintillation fluid was obtained from the Fisher Scientific Company.

Statistical analysis

Statistical significance of the data was determined by analysis of variance and followed by Scheffe's multiple range test. In all cases, identical or similar results were observed in at least three or more independent experiments. All results were presented as the mean ± SE of determinations of cells from quadruplicate cultures within each treatment group. A P value of less than 0.05 was considered significant.

III. Results

Effects of melittin, LHRH and arachidonic acid on progesterone production

To compare the effects of endogenous AA, exogenous AA-and LHRH on P_4 production, granulosa cells were treated with melittin, AA and LHRH for 5h (Fig. 41). Treatment of the cells with a maximal effective dose of melittin ($3x10^{-7}M$) caused a more than 2 fold increase in P_4 in the culture medium. In the same experiment, the degree of stimulation of P_4 production achieved with the maximal dose of exogenous AA and LHRH was significantly larger than that induced by melittin, 5.1 fold and 4 fold, respctively, as compared with the untreated control cells.

Sensitivity of progesterone response to arachidonic acid

The effects of increasing concentration of AA on P_4 production were determined 5h after the addition of AA to the granulosa cells (Fig. 42). A maximal enhancement of P_4 production was observed at AA concentrations above 10^{-5}M. The minimal effective dose of AA was $3x10^{-7}$M ($P<0.05$). In addition to enhancing P_4 accumulation in the culture medium, 10^{-5}M AA caused a significant increase in the intracellular P_4 concentration (0.53 ± 0.02 vs. 0.12 ± 0.01 ng/$2x10^5$ cells in control incubations; $P<0.01$).

Effect of unsaturated fatty acids on progesterone production

In this experiment, the specificity of other unsaturated fatty acids as well as AA action on P_4 production was investigated. All fatty acids were added to the culture medium at the concentration of 10^{-5}M. 11,14 eicosadienoic acid, homo-gamma-linolenic acid (C18:3), gamma-linolenic acid (C18:3), linoleic acid (C18:2) and AA increased P_4 production during a 5h incubation period. Interestingly, these fatty acids showed different potencies in stimulating P_4 production in the following order: AA> homo-gamma-linolenic> gamma-linolenic> linoleic> 11,14 eicosadienoic acid. Another unsaturated fatty acid, oleic acid, failed to stimulate P_4 production (Fig. 43).

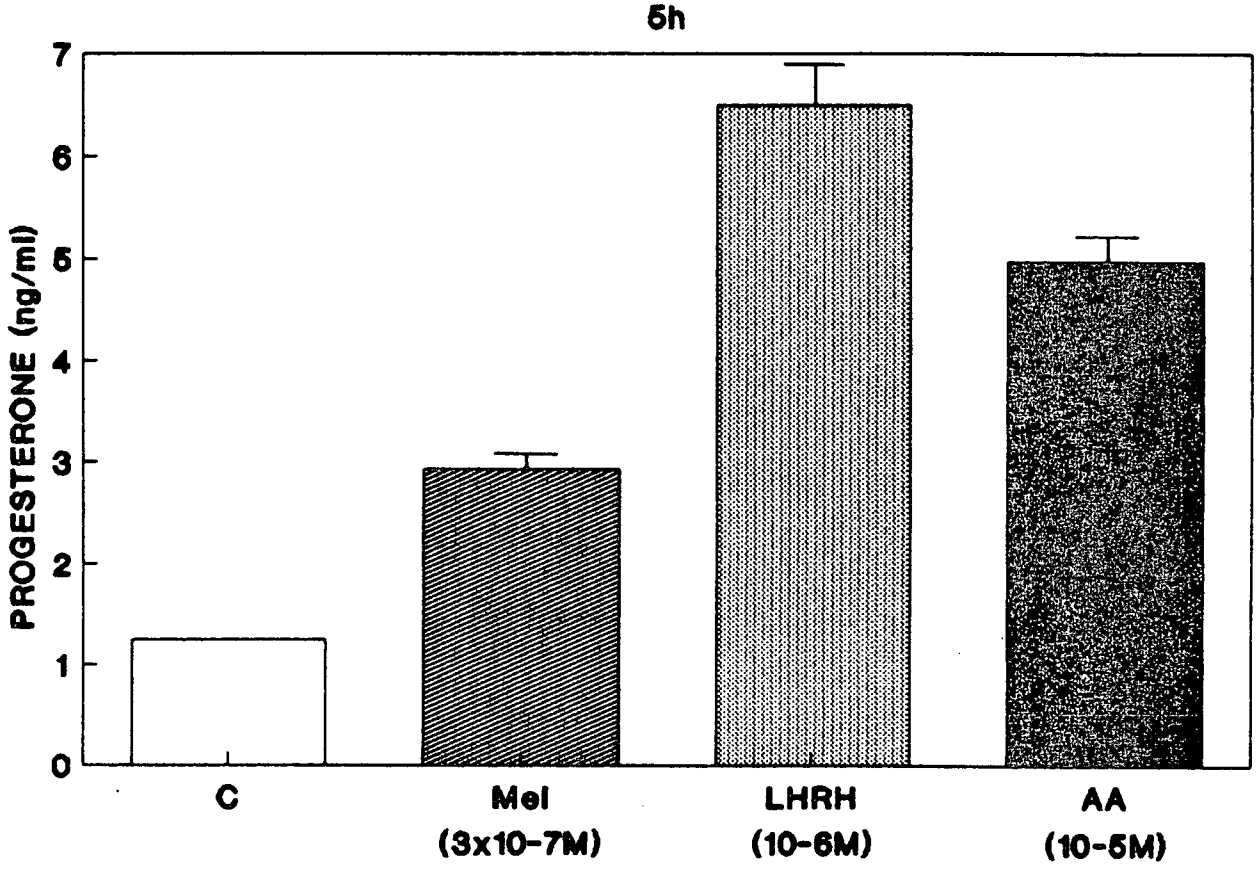

Fig. 41. Stimulatory effects of melittin, LHRH and arachidonic acid (AA) on progesterone (PROG) accumulation during a 5h culture period. C, control; Mel, melittin.

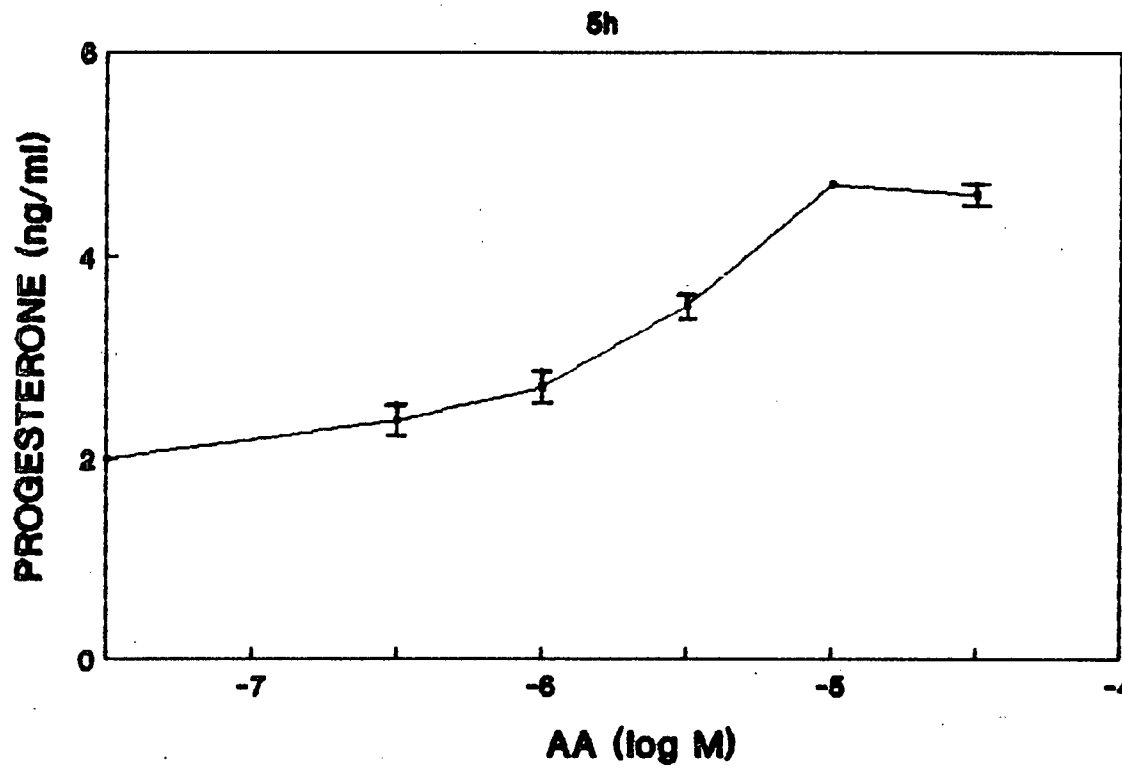

Fig. 42. Effect of increasing concentration of arachidonic acid (AA) on progesterone production during a 5h culture period. AA caused a dose dependent increase in progesterone production.

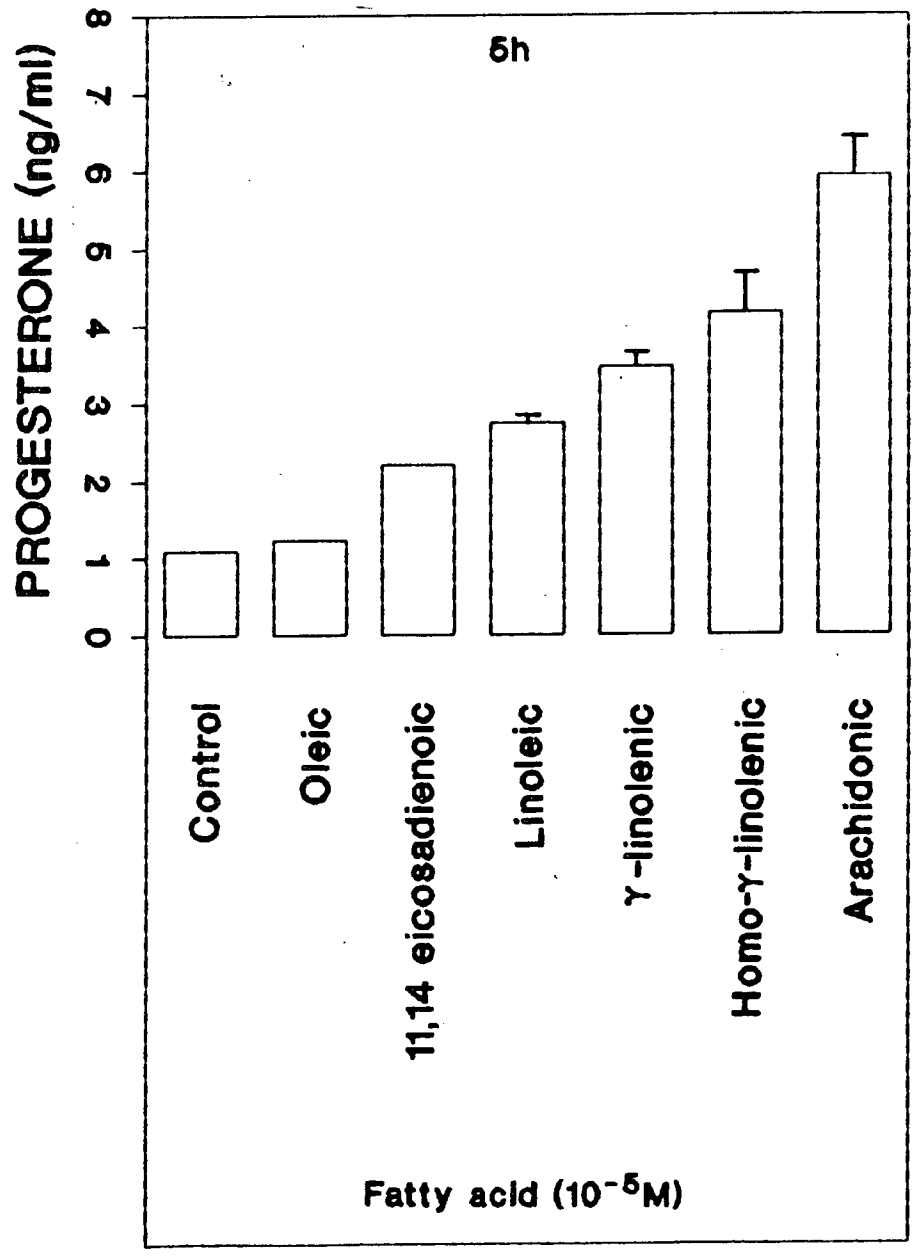

Fig. 43. Effects of unsaturated fatty acids on progesterone production. Arachidonic, homo-gamma-linolenic, gamma-linolenic, linoleic, and 11, 14 eicosadienoic acids stimulated progesterone production during a 5h culture period, whereas oleic acid failed to stimulate progesterone production.

Effects of LHRH and arachidonic acid on progesterone production

As expected, addition of LHRH (10^{-6}M) to rat granulosa cells enhanced P_4 production during a 5h incubation ($P<0.01$) (Fig. 44A). Addition of AA (10^{-5}M) also significantly enhanced the P_4 production to about 2.9 fold of the control level ($P<0.01$). The concomitant presence of LHRH and AA further stimulated P_4 levels to about 4.5 fold of the control P_4 values ($P<0.01$). As illustrated in Fig. 44 panel B, addition of AA to an agonistic analog of LHRH further enhanced P_4 production over that induced by the LHRH agonist alone ($P<0.01$).

Time course of effects of LHRH and arachidonic acid on progesterone production

As shown in Fig. 45, P_4 production was stimulated, 82% above the control ($P<0.05$), as early as 1h after the addition of 10^{-6}M LHRH. The effect of 10^{-5}M AA was somewhat slower in onset; by 3h after AA addition, P_4 production was increased significantly (1.9 fold) compared with that in control incubations ($P<0.05$). At 5h, the level of P_4 stimulated by AA was not different from that induced by LHRH. Interestingly, the concomitant presence of AA and LHRH at 1h did not further enhance P_4 production induced by LHRH alone ($P<0.05$), but markedly potentiated P_4 production at 3h and 5h ($P<0.01$) compared with that after treatment with AA or LHRH alone.

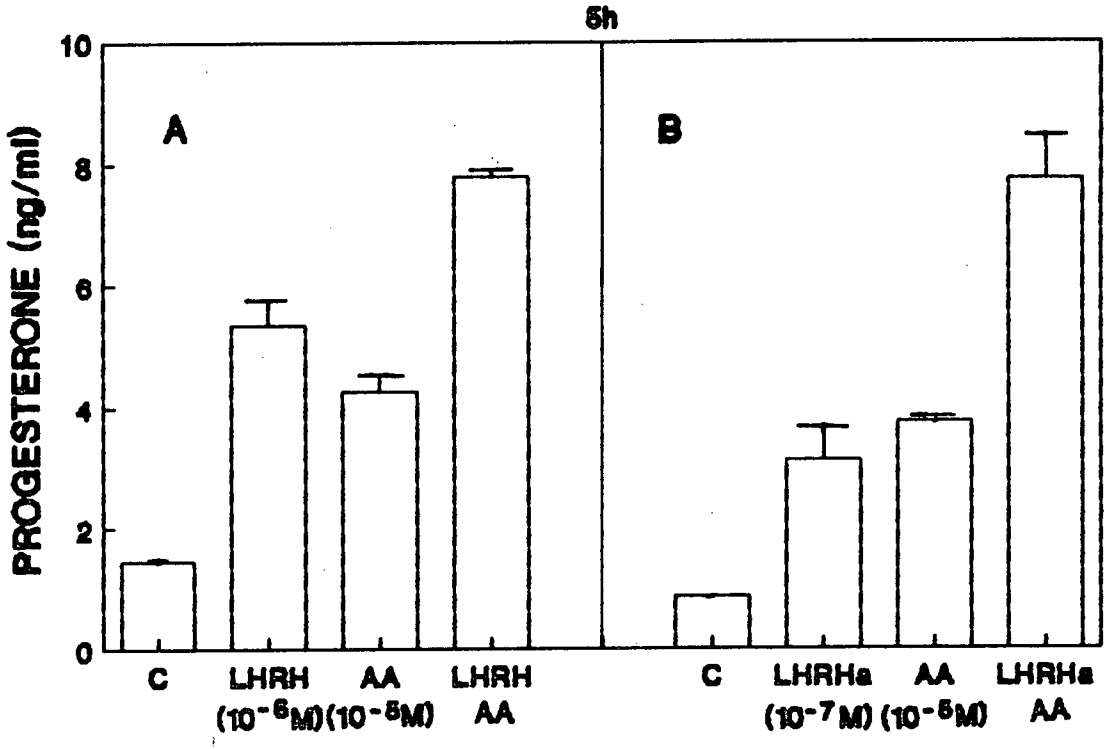

Fig. 44. Effects of treatment of granulosa cells with arachidonic acid (AA) and LHRH or a LHRH agonist (LHRHa) on progesterone production. LHRH (panel A) or LHRHa (panel B) stimulated progesterone production was further enhanced by AA during a 5h culture period.

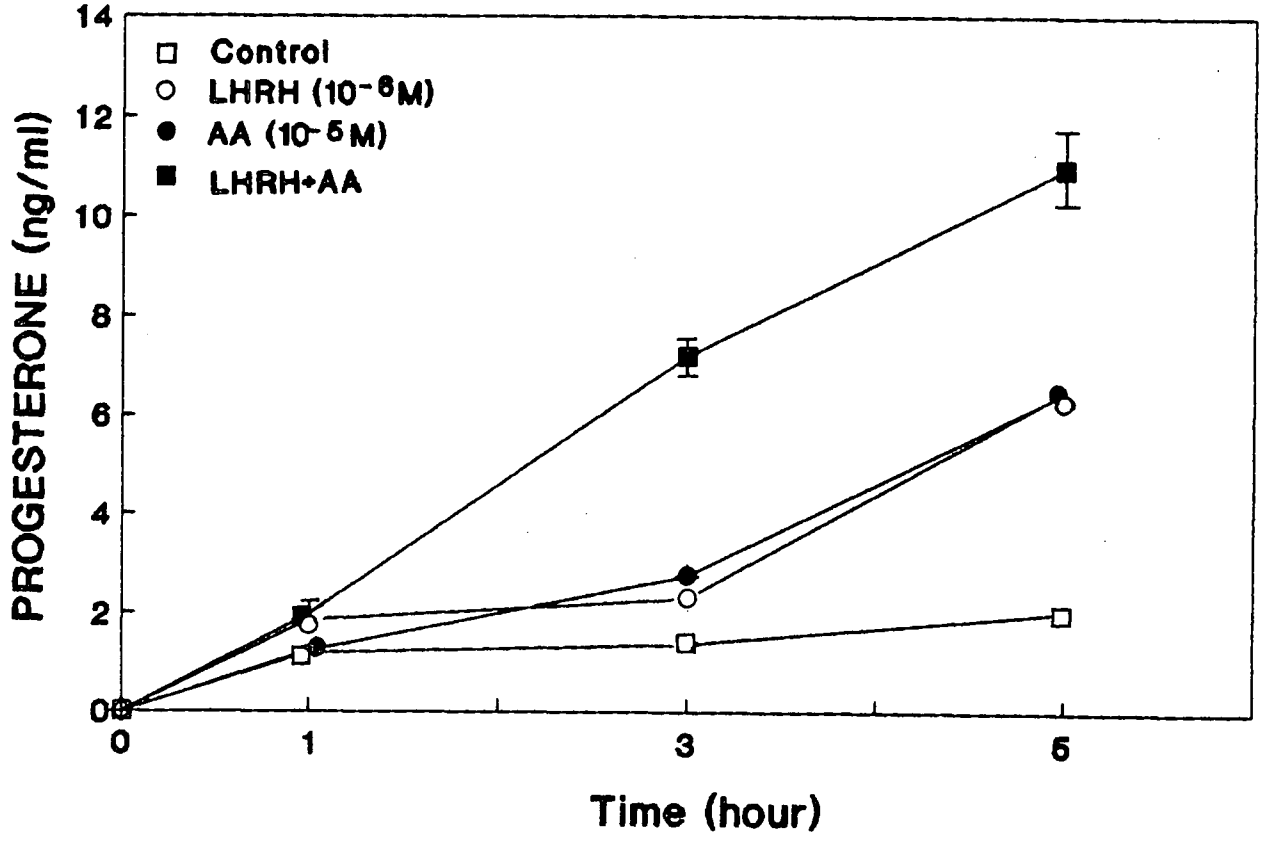

Fig. 45. Time course of stimulation of progesterone production by arachidonic acid (AA), LHRH or LHRH plus AA. Progesterone production was stimulated as early as 1h after the addition of LHRH, whereas the effect of AA was significant at 3h. Progesterone production was potentiated by the presence of both AA and LHRH at 3h and 5h compared with that after treatment with AA or LHRH alone.

Interaction between arachidonic acid and TPA on progesterone production

Addition of the phorbol ester, TPA (10^{-8}M), to granulosa cells resulted in a 93% increase in P_4 production (Fig. 46) as compared with untreated control cells. Additionally, the concomitant presence of TPA (10^{-8}M) and AA significantly enhanced ($P<0.01$) the stimulatory effect of AA (at 10^{-6}M or 10^{-5}M) on P_4 production. Likewise, as shown in Fig. 47, the addition of AA (10^{-5}M) to TPA-treated cells markedly potentiated the stimulation of P_4 production by TPA (at 10^{-9}, 10^{-8}, or 10^{-7}M) alone.

Role of arachidonic acid metabolism

To investigate the possible involvement of AA metabolites in P_4 production, granulosa cells were treated with indomethacin and NDGA with the presence of either LHRH or AA. As shown in Fig. 48, addition of the AA metabolism inhibitors alone (10^{-5}M) had a slight but significant ($P<0.05$) stimulatory effect on P_4 production. More importantly, addition of the same dose of NDGA partially suppressed (by about 50%) P_4 production induced by 10^{-6}M LHRH (Fig. 48, upper panel); the same molar concentration of indomethacin was ineffective. On the other hand, the concomitant presence of NDGA, but not indomethacin, inhibited AA-induced P_4 production to the same level as that caused by NDGA alone ($P<0.01$) (Fig. 48, lower panel).

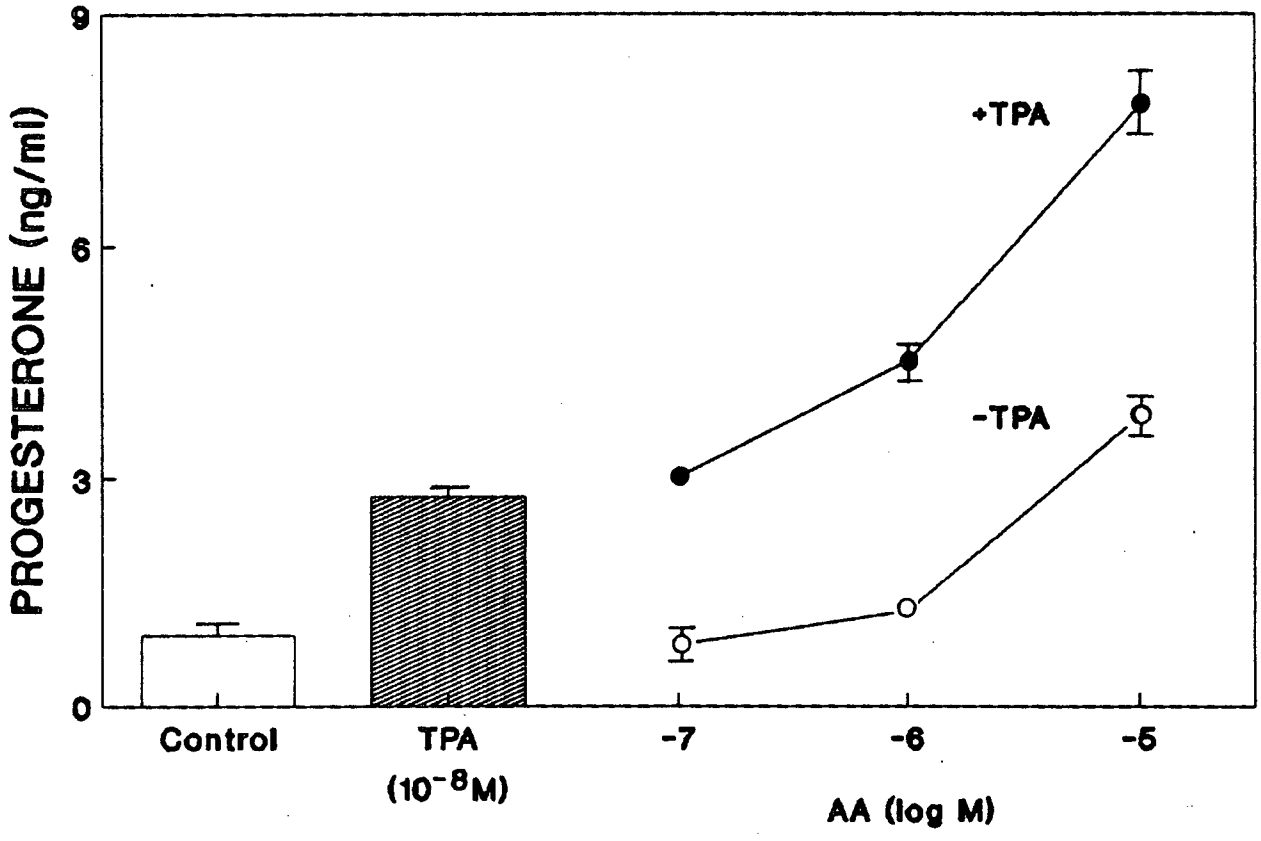

Fig. 46. Effects of the phorbol ester TPA and increasing concentrations of arachidonic acid (AA) on progesterone production. The presence of TPA enhanced the stimulatory effect of AA on progesterone production during a 5h culture period.

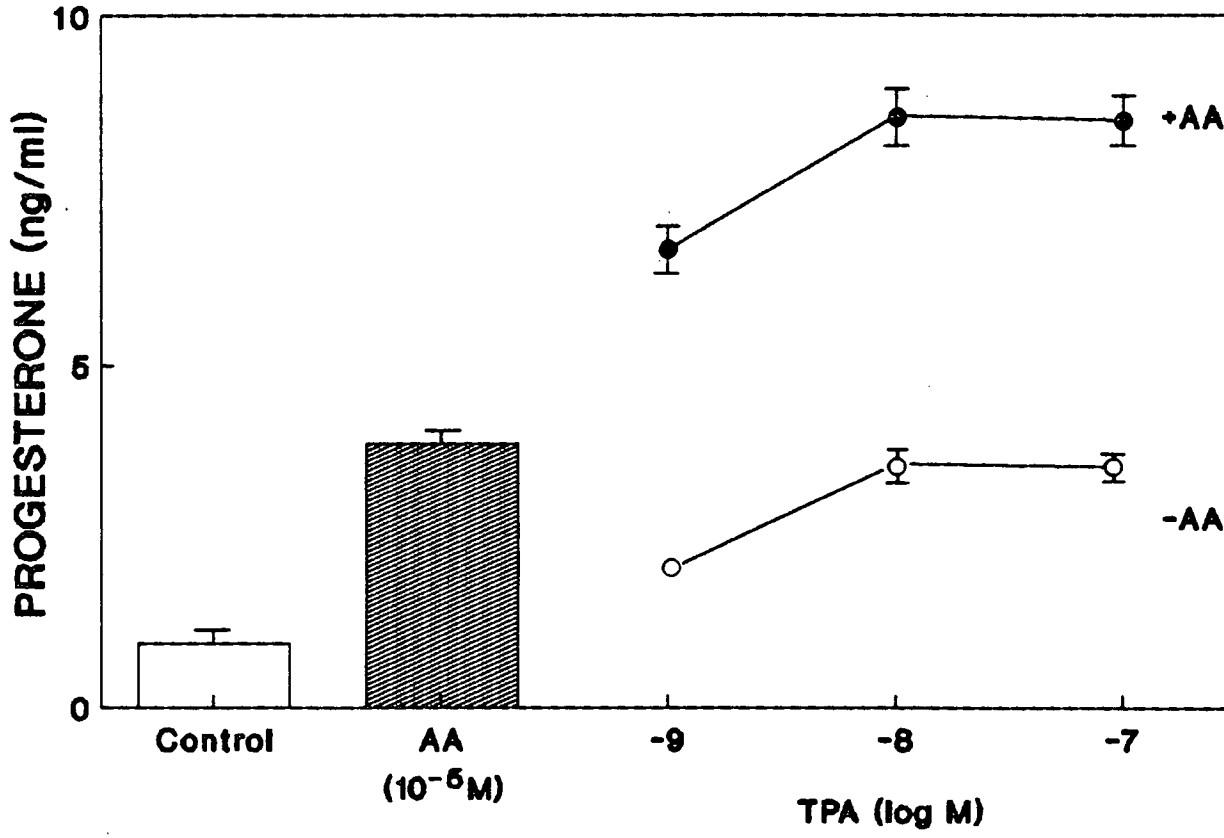

Fig. 47. Effects of arachidonic acid (AA) and increasing concentrations of the phorbol ester TPA on progesterone production. The addition of AA to TPA treated cells potentiated the stimulation of progesterone production by TPA alone during a 5h culture period.

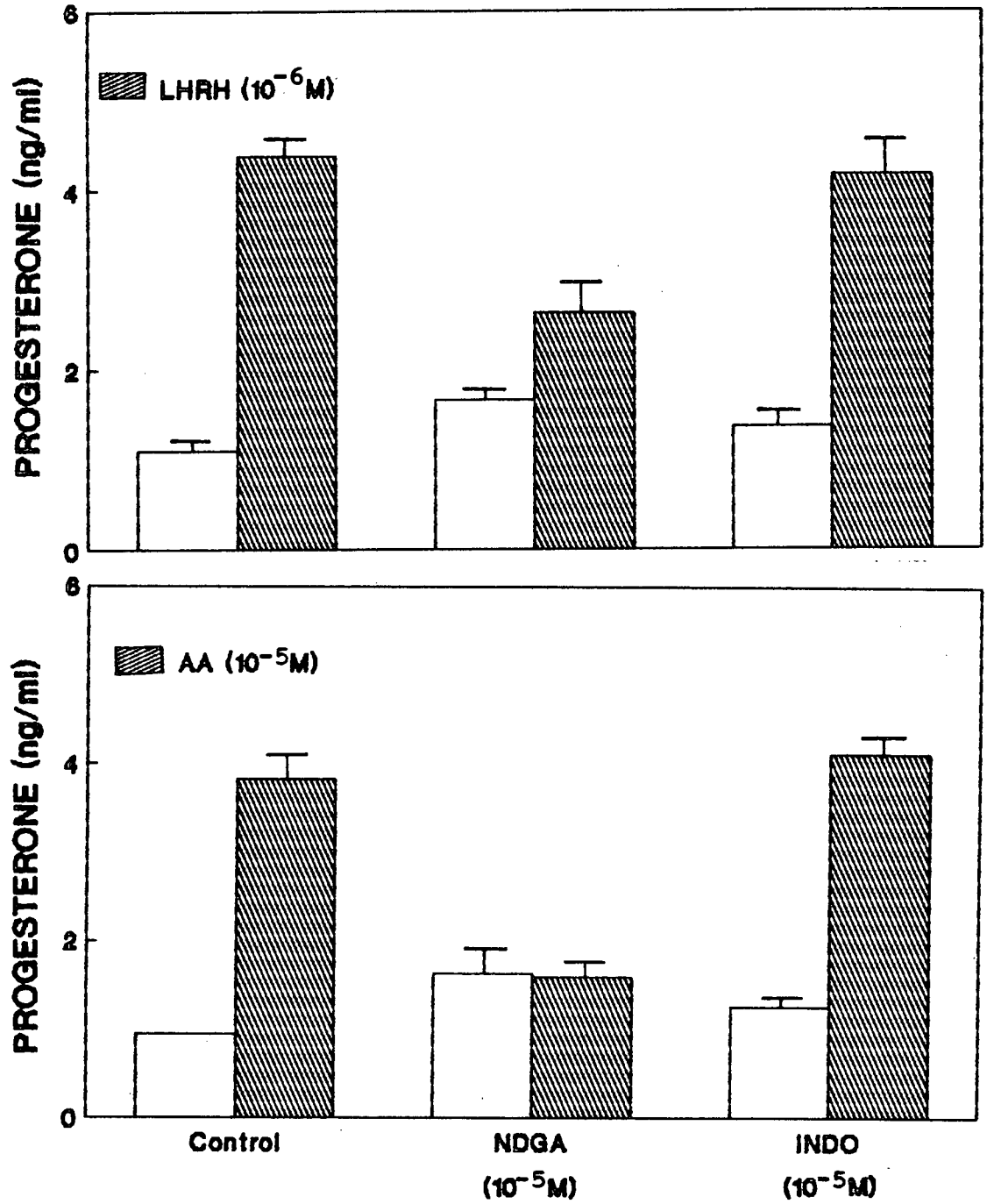

Fig. 48. Role of arachidonic acid (AA) metabolism. Addition of nordihydroguaiaretic acid (NDGA) partially suppressed progesterone production induced by LHRH (upper panel) and inhibited AA induced progesterone production to the same level as that caused by NDGA alone (lower panel), whereas indomethacin (INDO) was ineffective.

The effects of NDGA or indomethacin on the production of P_4 induced by LHRH plus AA were further examined in another experiment (Fig. 49). While indomethacin failed to affect the marked increase in P_4 production due to the concomitant presence of LHRH (10^{-6}M), AA (10^{-5}M) and NDGA (10^{-5}M) suppressed the P_4 response dramatically ($P<0.01$).

Dose response of HETEs and HPETEs on progesterone production

Since the lipoxygenase metabolites of AA may be involved in the action of LHRH on steroidogenesis, the effects of these lipoxygenase metabolites including hydroxyeicosatetraenoic acids (HETEs) and hydroperoxyeicosatetraenoic acids (HPETEs), on ovarian steroid hormone were further examined.

Rat granulosa cells were incubated for 5h in the absence or presence of increasing concentration of 5-HETE, 5-HPETE, 12-HETE, 15-HETE or 15-HPETE (10^{-7}M to 10^{-5}M). P_4 production was increased by these acids in a dose dependent manner. At 10^{-6}M, all treatments resulted in a slight but significant increase in P_4 formation. At 10^{-5}M, all compounds (except 15-HPETE) further stimulated P_4 production. The following order of potency was observed: 12-HETE > 5-HETE > 5-HPETE = 15-HETE > 15-HPETE (Fig. 50).

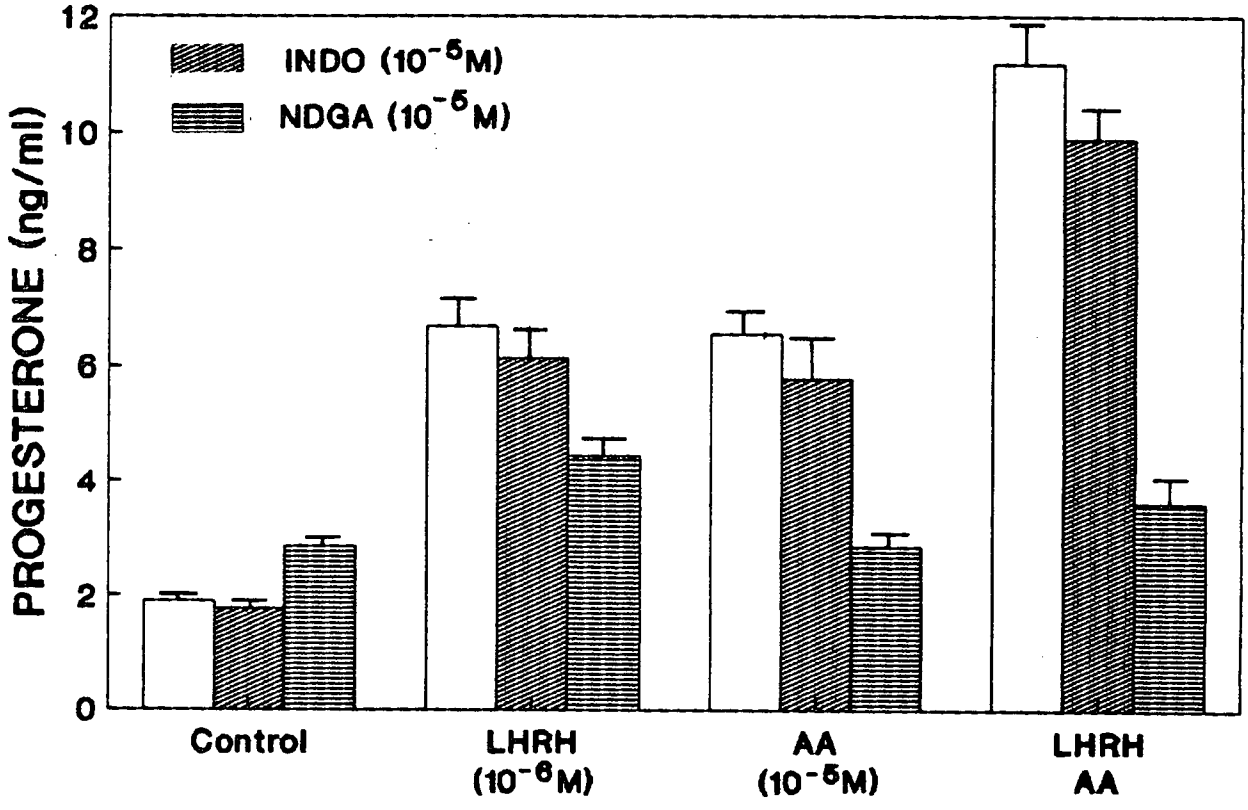

Fig. 49. Effects of nordihydroguaiaretic acid (NDGA) or indomethacin (INDO) on progesterone production induced by LHRH and/or arachidonic acid (AA). Whereas indomethacin did affect the increase in progesterone production due to the presence of both LHRH and AA, NDGA dramatically suppressed progesterone production.

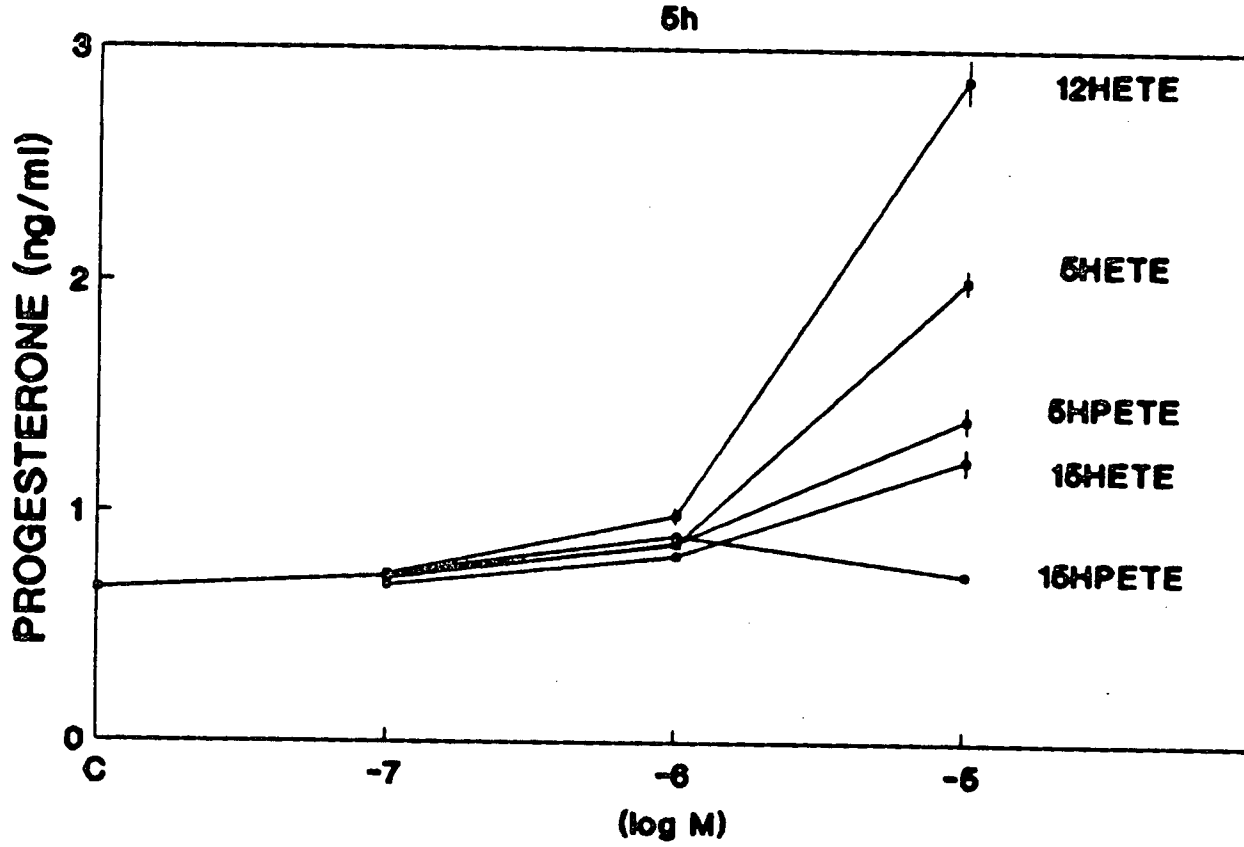

Fig. 50. Effects of HETEs and HPETEs on progesterone production. Progesterone production was increased by these fatty acids in a dose dependent manner during a 5h culture period.

Effects of HETEs on progesterone and PGE_2 production

Granulosa cells were treated with 5-, 12- or 15-HETE and the effects on P_4 as well as PGE_2 production were examined. As seen in Fig. 51 (upper panel), at $5x10^{-6}M$, 12-HETE was most potent and caused a 4.1 fold increase in P_4 formation. 5-HETE and 15-HETE resulted in 3.5 and 2.4 fold increase of P_4 accumulation, respectively, when compared with control incubations.

Interestingly, these AA metabolites also stimulated PGE_2 production in the same experiment (Fig. 51. lower panel). Unlike their actions on P_4 production, the effect of 15-HETE was as potent as 12-HETE on PGE_2 formation. 15-HETE or 12-HETE caused an approximate 15 fold increase in PGE_2 accumulation in the culture medium. In contrast, 5-HETE was considerably less potent when compared with 12- or 15-HETE, but still resulted in significant increase in PGE_2 formation, about 6 fold, when compared with the control incubations.

Interaction of HETEs or HPETEs with LHRH on progesterone and PGE_2 production

Since lipoxygenase metabolites of AA were believed to be involved in the action of AA, the effects of HETEs and HPETEs on the stimulation of P_4 production by LHRH were further investigated (Fig. 52, upper panel). At the minimum effective dose (i.e $10^{-6}M$), the AA metabolites stimulated basal P_4 production slightly. A more effective stimulation of P_4 was observed with $10^{-6}M$ LHRH. Concomitant treatment with LHRH and

the various AA metabolites caused further increase in P_4 accumulation, by 20% to 93%, when compared with the effect of LHRH alone.

PGE_2 production in the same experiments was also determined (Fig. 52, lower panel). 5-HETE and 5-HPETE, at the minimum effective dose which stimulated P, did not alter either basal or LHRH induced PGE_2 formation. In contrast, 12-HETE, 15-HETE or 15-HPETE significantly increased PGE_2 levels when compared with the control incubation. Furthermore, 12-HETE, 15-HETE and 15-HPETE augmented the stimulatory effect of LHRH on PGE_2 production by 2 fold, 2.9 fold and 2.5 fold, respectively, when compared with the LHRH treatment alone.

Interactions of HETEs or HPETEs with TPA on progesterone and PGE_2 production

The addition of the protein kinase C activator (TPA), at 10^{-7}M, to granulosa cells caused a marked increase in P_4 production (Fig. 53, upper panel). All HETEs and HPETEs tested significantly augmented the stimulatory effect of TPA, by 55 to 83%, when compared with P_4 levels induced by TPA alone.

In the same experiment, TPA alone caused a 4.9 fold increase in PGE_2 production(Fig. 53, lower panel). While 5-HETE or 5-HPETE did not significantly affect PGE_2 production induced by TPA, concomitant treatment with 12-HETE, 15HETE or 15-HPETE further enhanced TPA-stimulated PGE_2 accumulation.

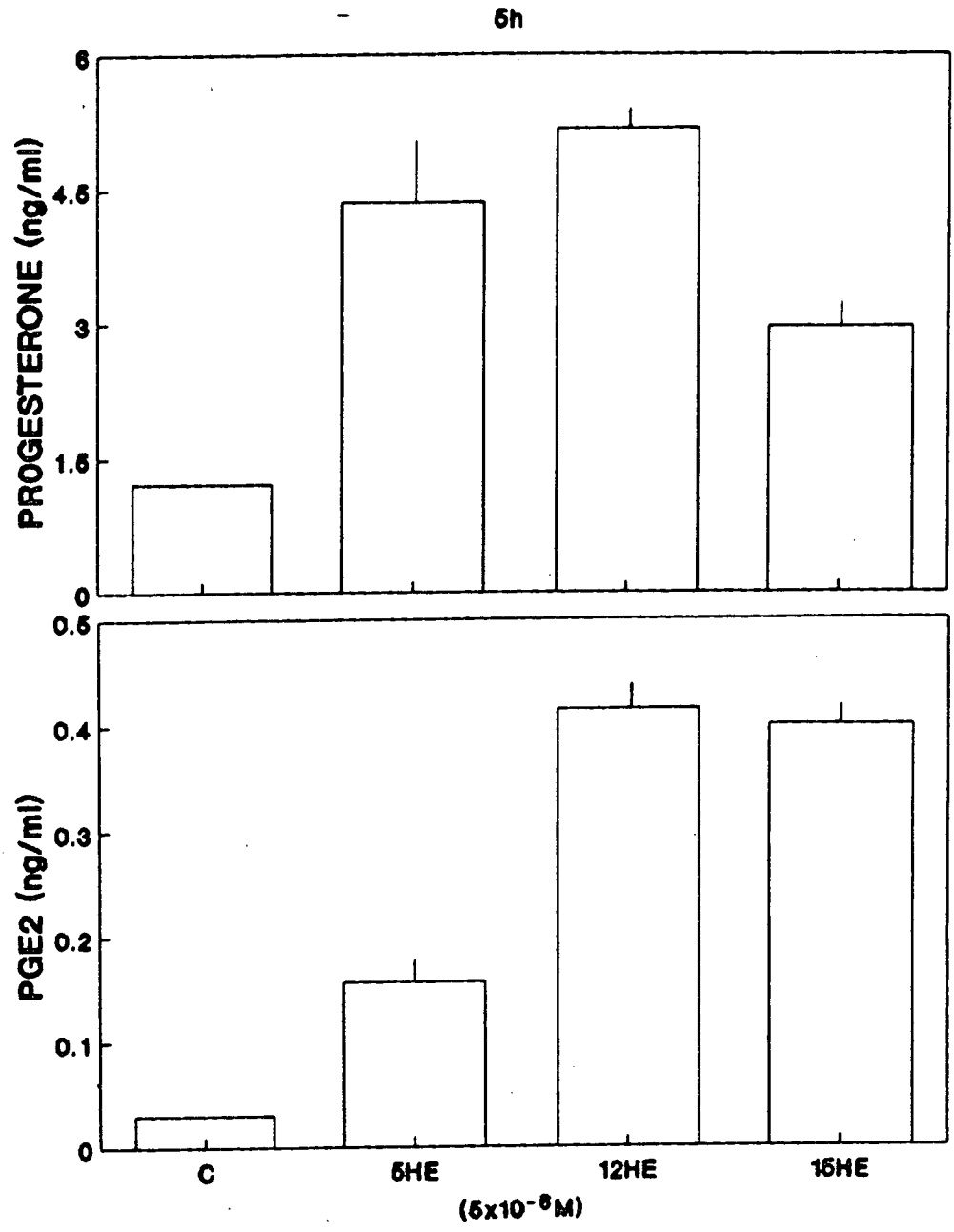

Fig. 51. Effects of HETEs on progesterone (upper panel) and PGE_2 (lower panel) production. Both progesterone and PGE_2 were stimulated by HETEs during a 5h culture period. 5HE, 5-HETE; 12HE, 12-HETE; 15HE, 15-HETE.

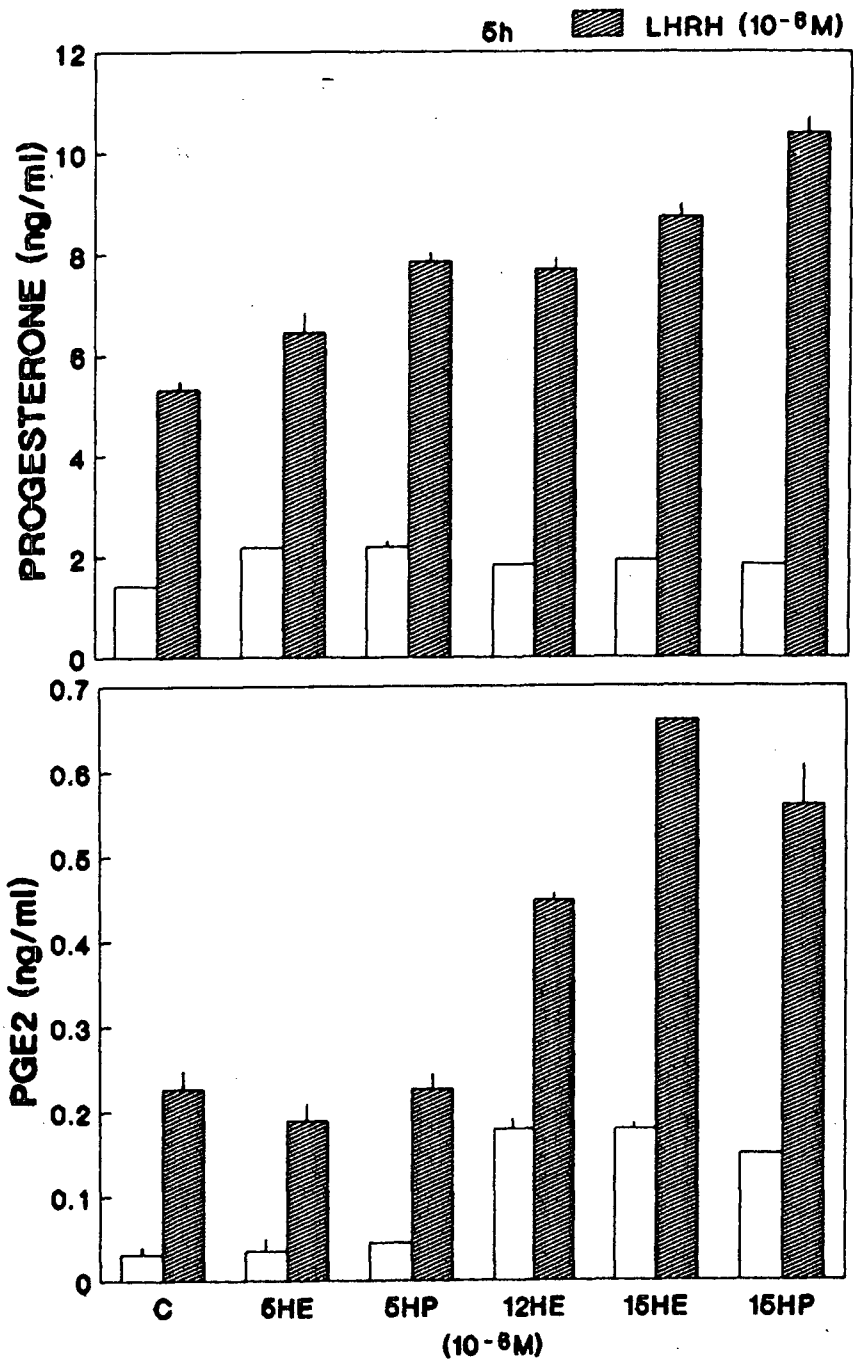

Fig. 52. Interactions of HETEs or HPETEs with LHRH on progesterone (upper panel) and PGE_2 (lower panel) production. At the minimum effective dose (10^{-8} M), the various arachidonic acid metabolites enhanced LHRH induced progesterone production and 12-HETE, 15-HETE and 15-HPETE augmented the stimulatory effect of LHRH on PGE_2 production during a 5h culture period.

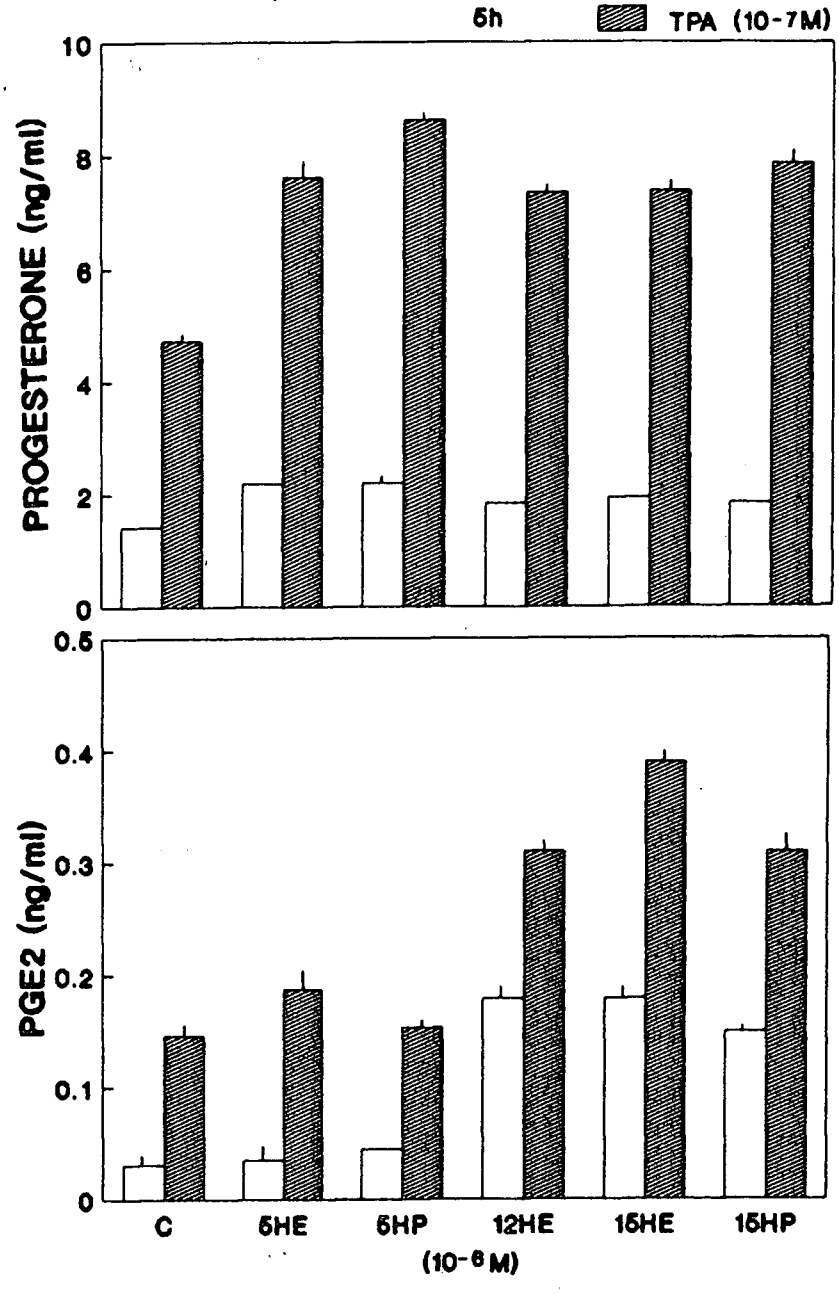

Fig. 53. Interactions of HETEs or HPETEs with the phorbol ester TPA on progesterone (upper panel) and PGE_2 (lower panel) production. TPA induced progesterone production was enhanced by all HETEs and HPETEs tested, while TPA induced PGE_2 production was augmented by 12-HETE, 15-HETE and 15-HPETE during a 5h culture period.

Effect of LHRH on FSH-induced progesterone production: time response

To examine the action of gonadotrophin and LHRH on granulosa cells, FSH- or LHRH- or FSH plus LHRH-treated granulosa cells were cultured for 8h, 16h and 24h (Fig. 54). FSH (100 ng) alone resulted in a significant time dependent increase in P_4 accumulation, 20 fold, 21.5 fold and 30 fold, at 8h, 16h and 24h, respectively. LHRH alone also markedly stimulated P_4 production as compared with untreated culture cells, but LHRH-induced P_4 production was much less than that induced by FSH. The concomitant presence of FSH with LHRH in the culture medium did not alter P_4 production at 8h. However, a significant decrease in P_4 production was observed at 16h, and P_4 production was further reduced at 24h in combined treatment of granulosa cells with FSH plus LHRH.

Effects of arachidonic acid and/or FSH on progesterone production during a 24h culture

To examine the role of AA on P_4 production, rat granulosa cells were treated with 10^{-5}M AA, in the absence or presence of FSH (100ng), for 24h (Fig. 55). As expected, FSH markedly stimulated P_4 production (23 fold) compared with the untreated control ($P<0.01$). AA alone caused a slight but significant stimulation of P_4 production, 4.1 fold ($P<0.05$) when compared with the untreated control cells. The concomitant presence of AA with FSH did not affect FSH-induced increase in P_4 accumulation.

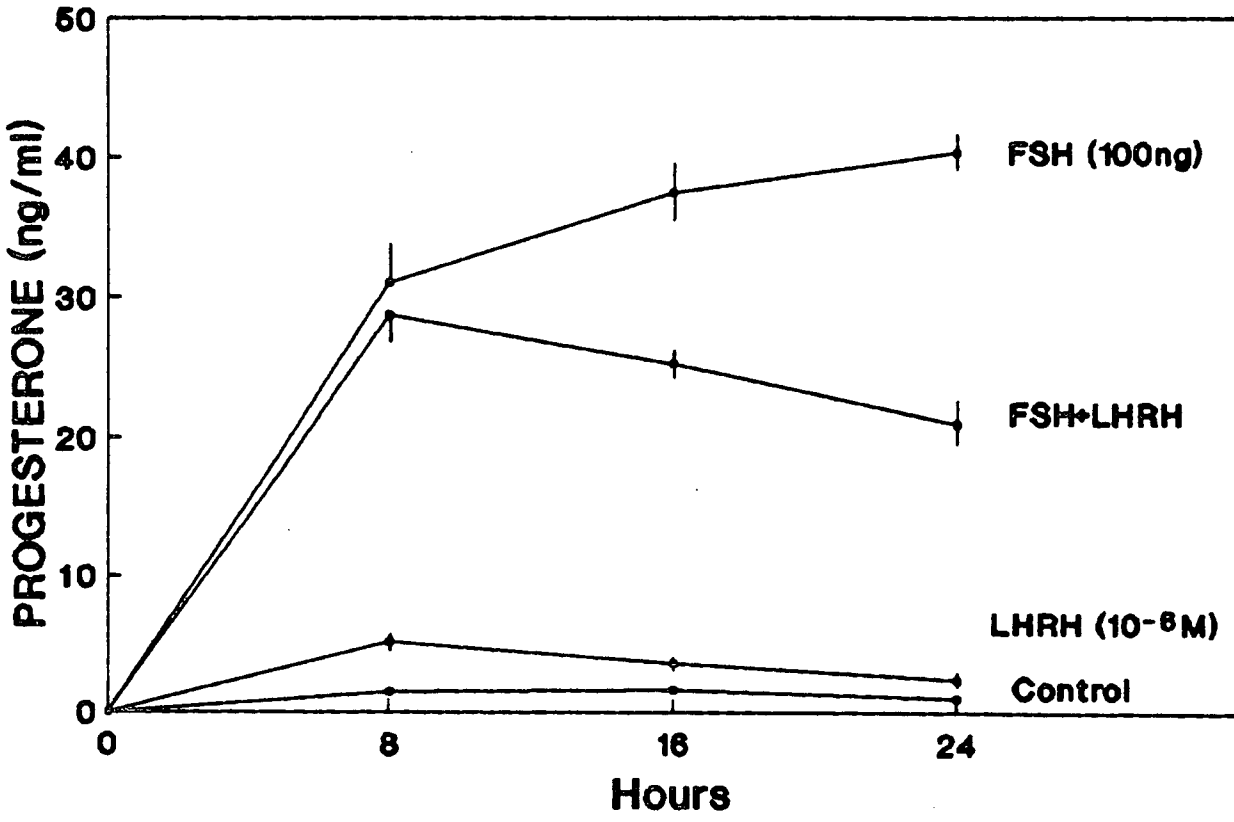

Fig. 54. Effect of LHRH on FSH induced progesterone production: time response. LHRH alone stimulated progesterone production as early as 4h, but production was much less than that induced by FSH. FSH stimulated progesterone production was reduced by LHRH after 16h.

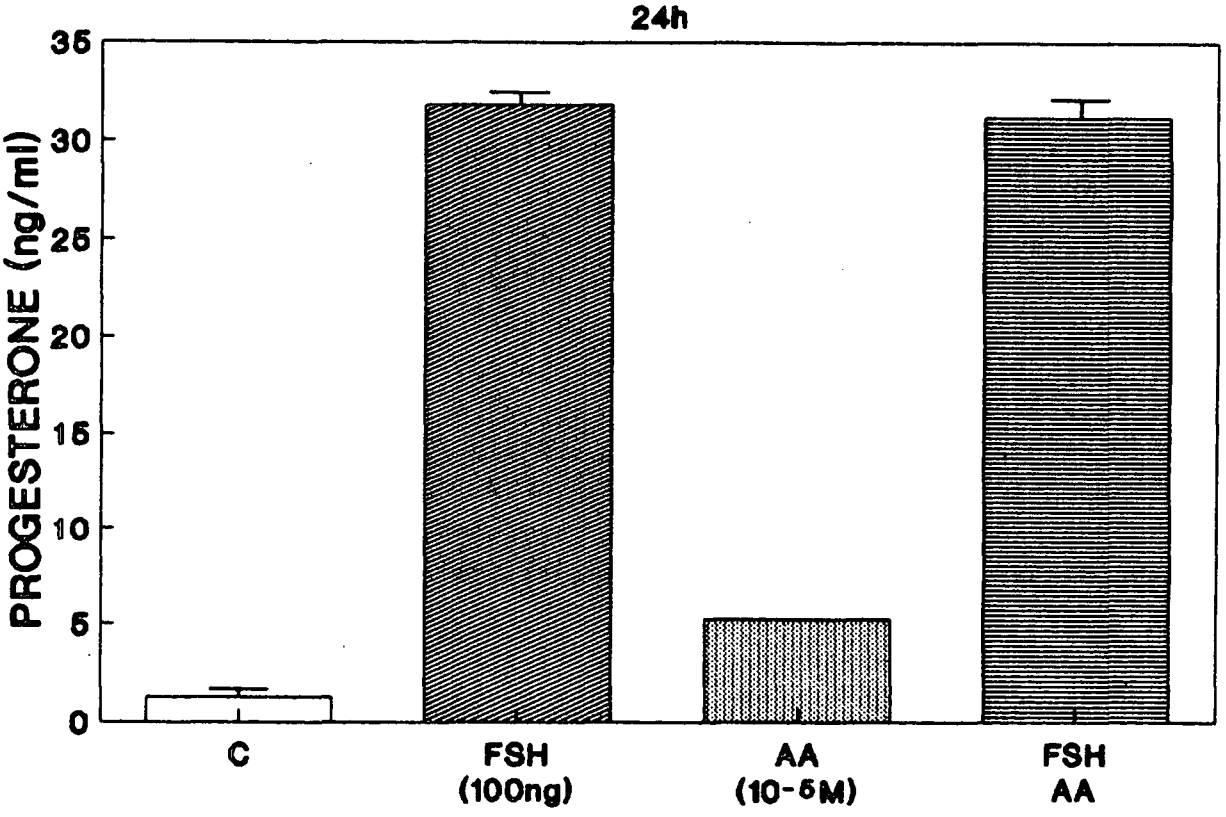

Fig. 55. Effects of arachidonic acid (AA) and/or FSH on progesterone production. AA alone caused a slight increase in progesterone production. Concomitant presence of AA with FSH did not affect the FSH induced increase in progesterone production after 24h culture. Control (C) and FSH treated cells received the appropriate amount of solvent for AA.

Effect of arachidonic acid on LHRH induced inhibition of progesterone production

Granulosa cells were treated for 18h with FSH (100 ng/ml), with or without the presence of LHRH (10^{-6}M). At the end of 18h, the combined treatment of LHRH plus FSH significantly decreased P_4 production when compared with the cells given FSH alone. At this time, AA was added to two of the groups and the culture was continued for a further 6h. As shown in Fig. 56, LHRH decreased FSH-induced P_4 production, by 47%, at the end of the 24h incubation period. When AA was present during the last 6h, the inhibitory effect of LHRH on FSH-induced P_4 was partially reversed, by about 42%, when compared with the cultured cells treated with LHRH plus FSH. AA by itself did not affect P_4 accumulation induced by FSH during the last 6h.

Effect of arachidonic acid on TPA-induced inhibition of progesterone production

Fig. 57 illustrates AA reversal of the inhibitory action of TPA on P_4 production. Granulosa cells were treated with FSH (100 ng/ml) with or without 10^{-9}M TPA for 18h. After that, AA was given to one of the groups. All incubations were continued for another 6h. As expected, TPA caused a marked inhibition on FSH-induced P_4 production, 69% ($P<0.01$) as compared with the untreated group. Addition of AA to the group given TPA plus FSH resulted in a partial reversal of P_4 production by 71%, when compared with the TPA plus FSH treatment alone ($P<0.01$).

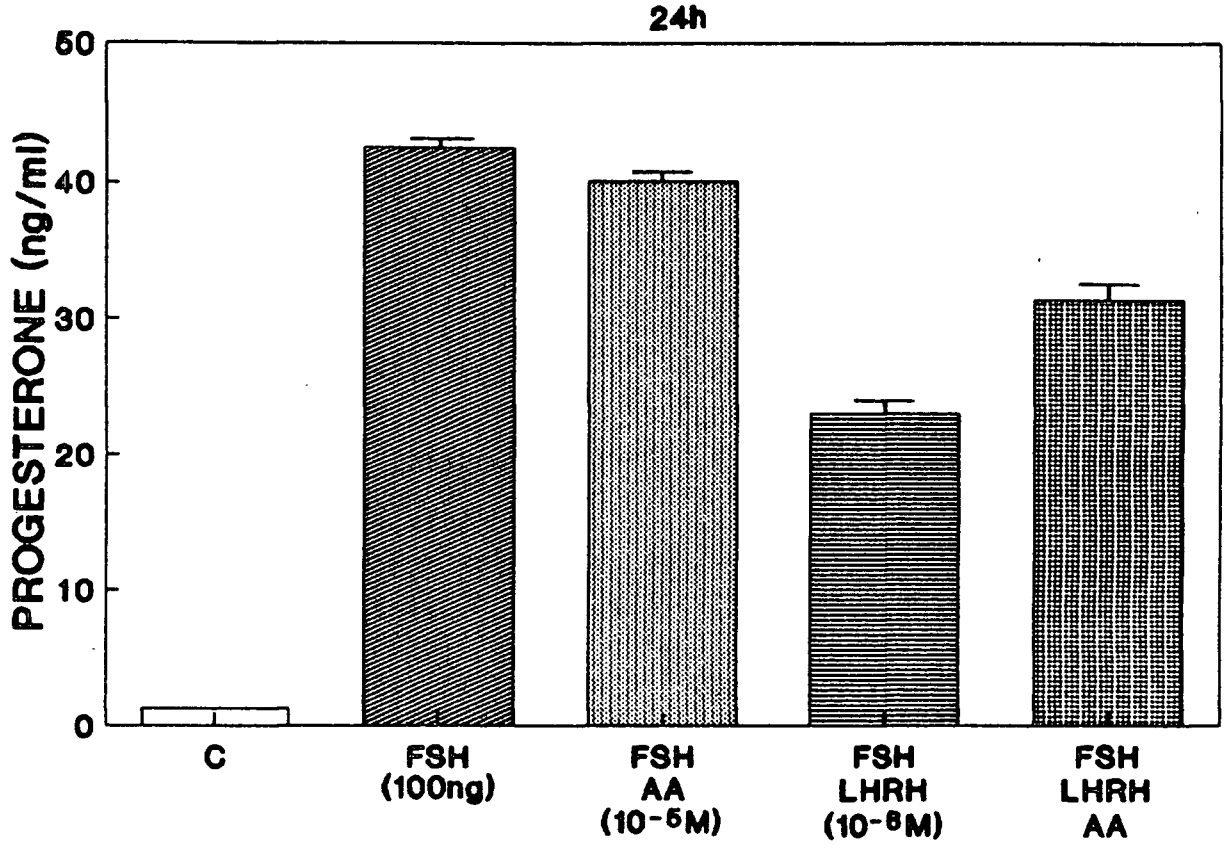

Fig. 56. Response to arachidonic acid (AA) after pretreatment with FSH and LHRH. Granulosa cells pretreated with saline (C) or with FSH ± LHRH for 18h, at which time AA (or solvent) was added. All groups were incubated for a further 6h. The inhibitory effect of LHRH on FSH induced progesterone production was partially reversed by the presence of AA.

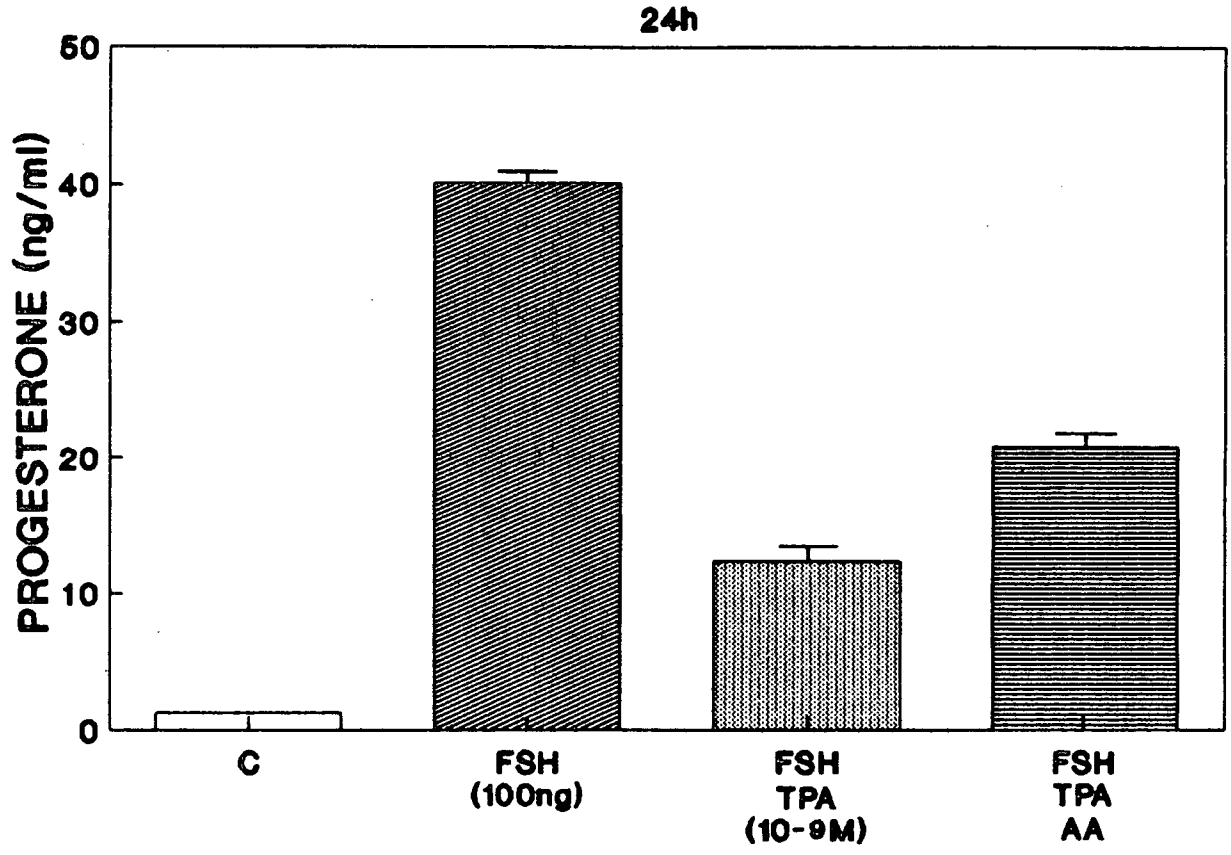

Fig. 57. Response to arachidonic acid (AA) after pretreatment with FSH and the phorbol ester TPA. Granulosa cells were pretreated with DMSO (C) or with FSH ± TPA for 18h prior to the addition of AA (or solvent). Then, the cells were incubated for 6h. TPA caused inhibitory effect on progesterone production by FSH was partially reversed by the addition of AA.

Response to arachidonic acid after pretreatment with cholera toxin and TPA

Addition of CT resulted in a 29 fold increase in P_4 production and TPA significantly attenuated the production of P_4 induced by CT. AA was added to two groups that had been precultured with CT or CT plus TPA for 18h. All incubations were continued for a further 6h. As shown in Fig. 58, the presence of AA caused a partial reversal, i.e. 39% ($P<0.01$) increase in P_4 production, when compared with the treatment with TPA plus CT alone. CT-induced P_4 production was not significantly affected by the addition of AA alone during the last 6h culture period.

Effect of arachidonic acid on P_4 production after pretreatment with TPA and LHRH

10^{-9}M TPA and 10^{-6}M LHRH were added to granulosa cells at the beginning of the culture in the absence of exogenous gonadotropins or CT. After 18h, AA was added to some of the groups for a further 6h to determine the P_4 response of the cells. At the end of the 24h culture, TPA did not stimulate P_4 production (Fig. 59). In contrast, LHRH caused a 55% ($P<0.01$) increase in P_4 accumulation when compared with untreated controls. Addition of AA alone during the last 6h resulted in a 160% increase in P_4 formation. When AA was added to the TPA-pretreated cells, P_4 production was same as that caused by AA alone. In contrast, when AA was given to the LHRH-pretreated cells, there was an additive effect on P_4 production.

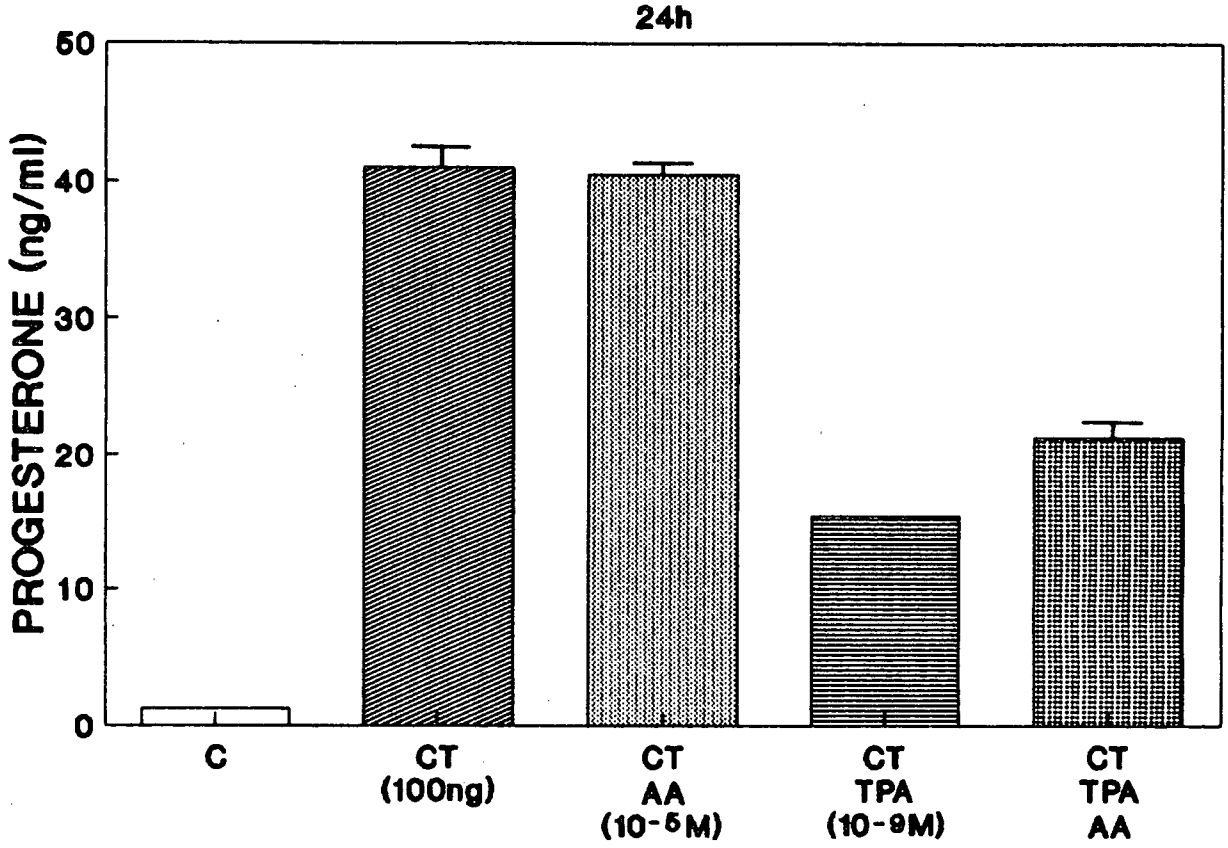

Fig. 58. Response to arachidonic acid (AA) after pretreatment with cholera toxin (CT) and the phorbol ester TPA. Granulosa cells were pretreated with DMSO (C) or with CT ± TPA. At 18h, AA (or solvent) was added. All incubations were stopped 6h later. The presence of AA partially reversed the inhibitory effect of TPA on CT induced progesterone production.

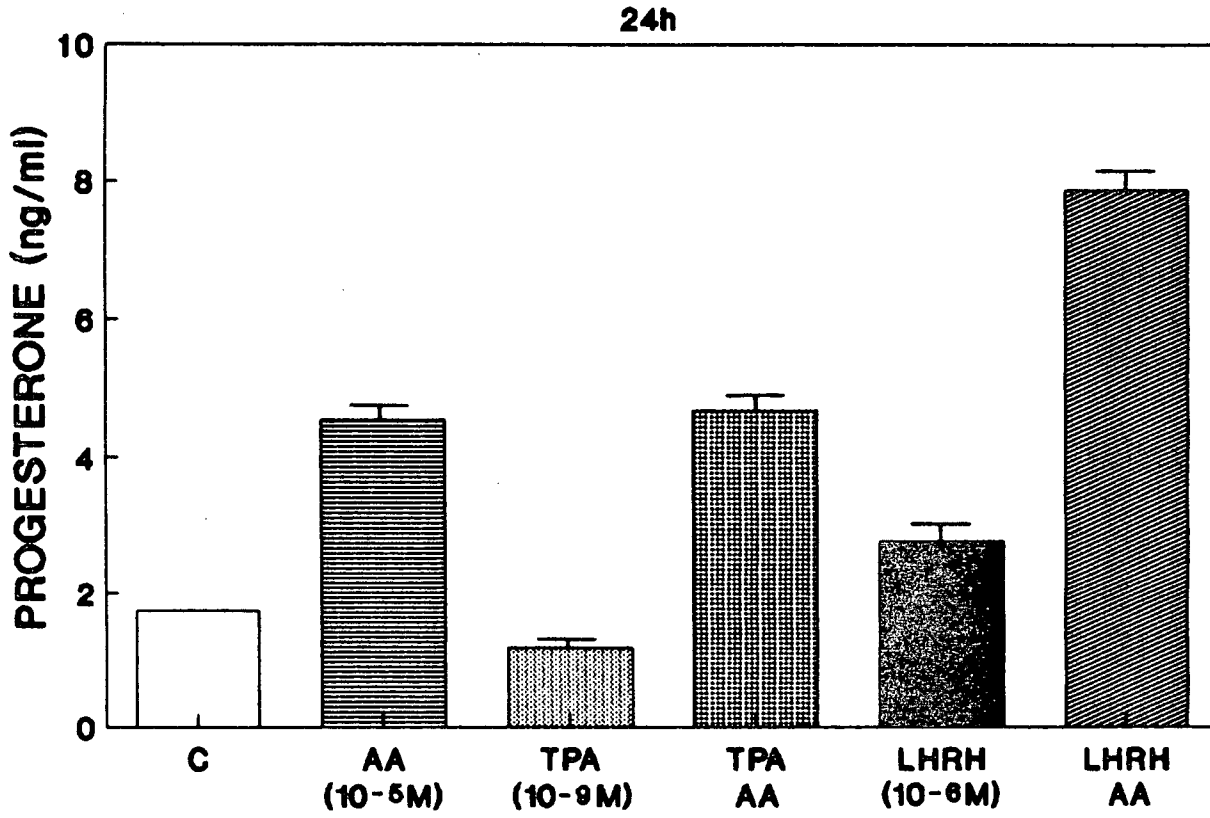

Fig. 59. Response to arachidonic acid (AA) after pretreatment with the phorbol ester TPA and LHRH alone. Granulosa cells were pretreated with dimethylsulfoxide (C), TPA or LHRH for 18h. At this time, AA (or solvent) was added. All groups were incubated for a further 6h. Treatment of the cells with TPA did not affect the response to AA.

Effects of LHRH, TPA and/or arachidonic acid on progestin production during a 5h incubation

Granulosa cells were treated for 5h with TPA (10^{-7}M) or LHRH (10^{-6}M), with or without the concomitant presence of AA (10^{-5}M). As shown in A panel of Fig. 60, the presence of AA, TPA or LHRH alone caused significant increases in P_4 production, by 4.8 fold, 7.8 fold and 7.1 fold, respectively, when compared with control levels. AA exerted an additive effect with TPA and LHRH on P_4 formation.

The production of 20alpha-OH-P is shown also in Fig. 60 (B panel). AA, TPA and LHRH stimulated 20alpha-OH-P production by 1.5 fold, 4.9 fold and 4.7 fold, respectively. The magnitude of AA-induced 20alpha-OH-P accumulation was much lower than that induced by either TPA or LHRH. In contrast to the additive effects observed for P_4 formation, AA did not alter the effect of TPA or LHRH on the accumulation of 20alpha-OH-P. On the other hand, total progestin accumulation (i.e. P_4 plus 20alpha-OH-P) was increased by treatment with AA, TPA or LHRH alone and further increased by combined treatment with TPA plus AA, or with LHRH plus AA (Fig. 60, C panel).

Effects of arachidonic acid, TPA and LHRH on 25-hydroxycholesterol enhanced steroidogenesis during a 5h incubation

To determine if AA affects the activity of the side-chain cleavage enzyme, a water soluble cholesterol derivative, 25-OH-cholesterol, was used. Inclusion of 25-OH-cholesterol in the

culture medium significantly enhanced the accumulation of P_4 in the control cells by 4.1 fold during a 5h incubation (Fig. 61). The concomitant presence of TPA or LHRH with 25-OH-cholesterol markedly increased P_4 production by about 56% and 84% ($P<0.01$) respectively, when compared with P_4 formation by 25-OH-cholesterol alone. The presence of AA with 25-OH-cholesterol also significantly increased P_4 production, about 34%, when compared to the effect of 25-OH-cholesterol alone. Nevertheless, AA failed to further enhance TPA- and LHRH-stimulated P_4 production in the presence of 25-OH-cholesterol, which was different from the effect of AA on P_4 production without the added cholesterol.

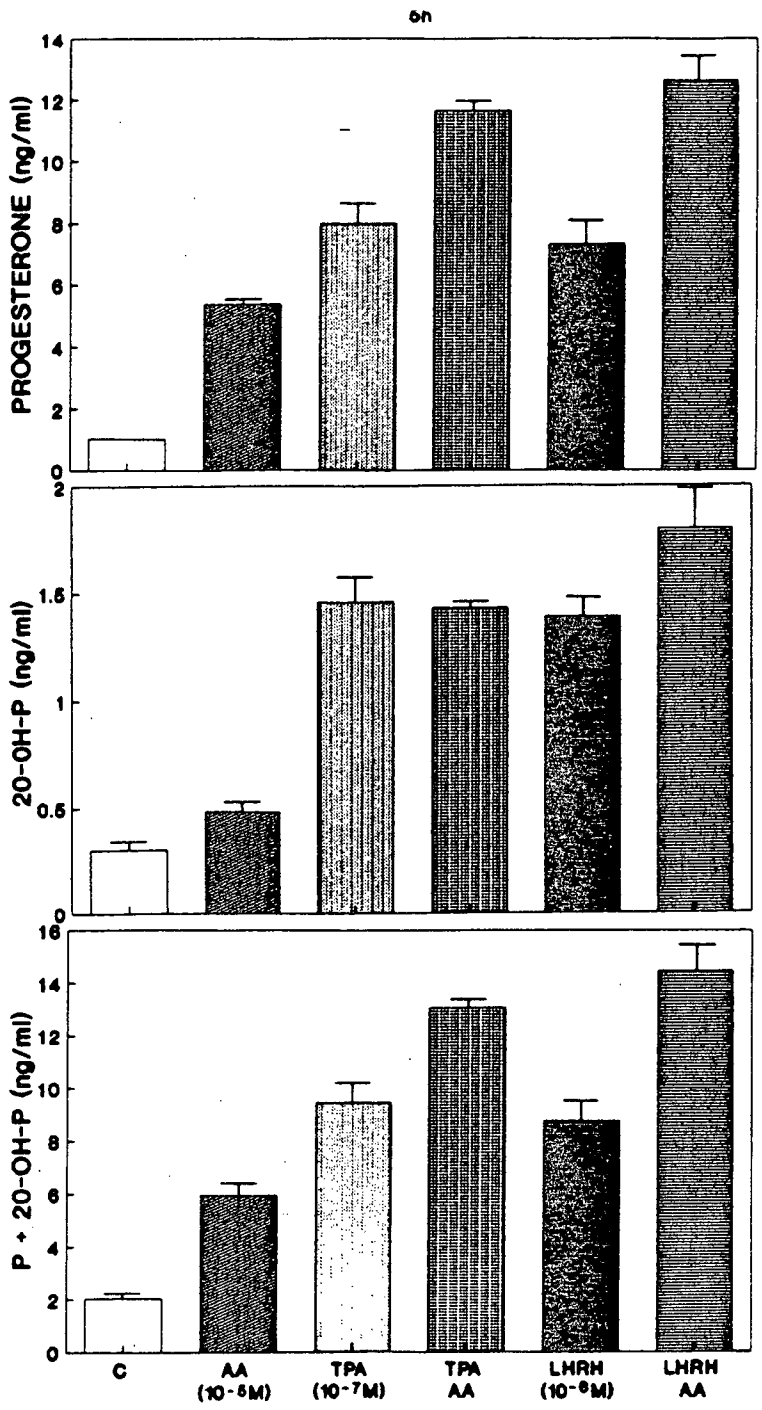

Fig. 60. Effects of LHRH, the phorbol ester TPA and/or arachidonic acid (AA) on progestin production during a 5h incubation. AA, TPA or LHRH alone caused significant increase in progesterone production, and AA exerted an additive effect with TPA and LHRH, whereas the magnitude of AA induced 20-alpha-OH-P accumulation was much lower than that induced by either TPA or LHRH, and AA did not alter the effect of TPA or LHRH on the accumulation of 20-alpha-OH-P.

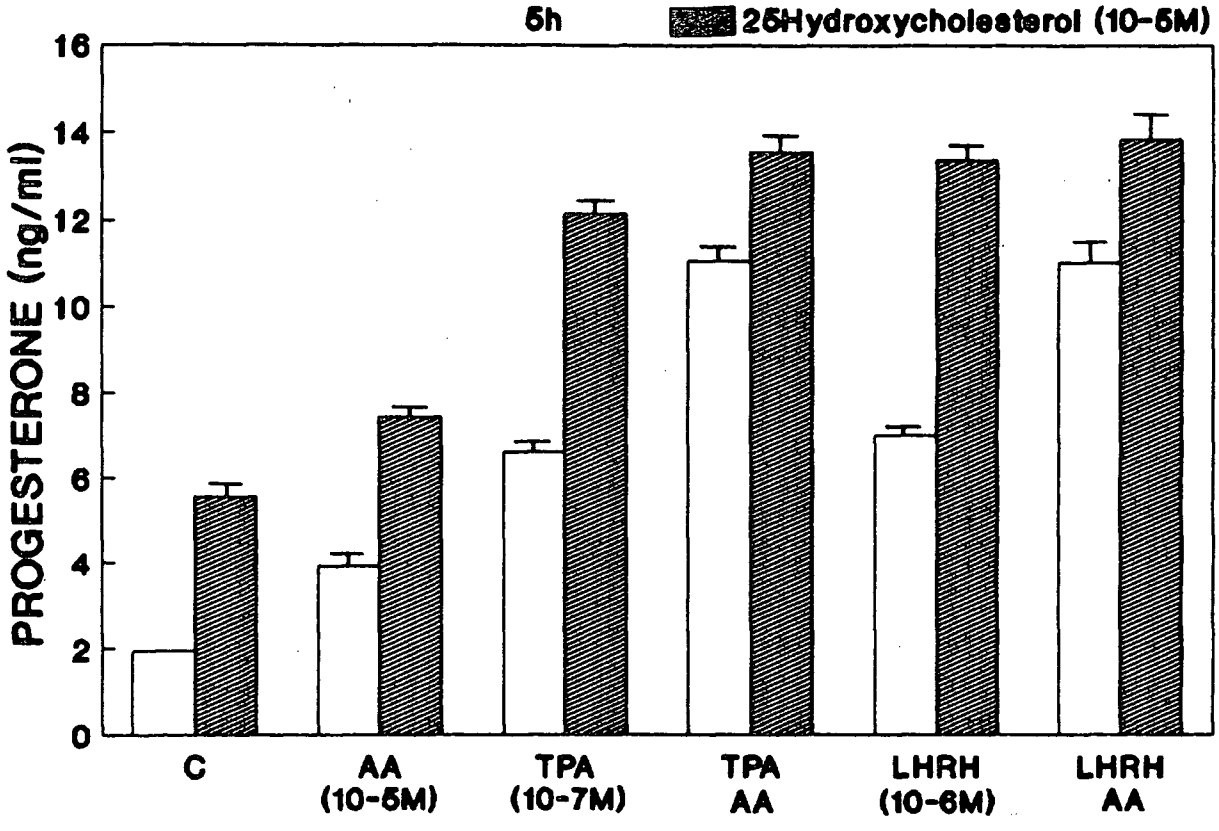

Fig. 61. Effect of arachidonic acid (AA), the phorbol ester TPA and LHRH on 25-hydroxycholesterol enhanced steroidogenesis during a 5h incubation. Although the presence of AA increased 25-OH-cholesterol induced progesterone production, AA failed to further enhance TPA and/or LHRH stimulated progesterone production in the presence of 25-OH-cholesterol. Incubations containing 25-hydroxycholesterol were denoted by the hatched bars.

IV. Discussion

The intracellular pathway by which LHRH stimulated AA release in granulosa cells was not clear. Three possible mechanisms for the liberation of AA from plasma membrane have been proposed (chapter 2). The increase of cellular level of free or unesterified radiolabeled AA was observed by treatment of granulosa cells with LHRH within 5 min (chapter 2). This observation strengthens the previous proposal that LHRH stimulated AA liberation from phospholipids might also be involved as an early step in LHRH signal transduction in the ovarian cells (Minegishi and Leung, 1985; chapter 2). It has also been shown that AA release in granulosa cells is enhanced by the calcium ionophore A23187 (Kawai and Clark, 1986; Minegishi et al., 1987), and potentiated by TPA (chapter 2), suggesting that LHRH-induced AA release is calcium dependent, and is regulated by the activation of PKC. Nevertheless, it appears that in many tissues a single extracellular signal could induce activation of both phospholipase C and phospholipase A_2 reactions and as a result, cause AA release from various phospholipids (Lapetina, 1982).

In the present study the effects of melittin, AA and LHRH on P_4 production were further examined. Melittin could induce P_4 production, but melittin stimulated P_4 production was lower than exogenous AA and LHRH stimulated P_4 formation in the same experiment (Fig. 41). This may reflect the fact that the quantity of endogenous AA was limited. Additionally, the results suggested that the effect of LHRH cannot be only due to

endogenous AA and its metabolites, and that Ca^{2+} and PKC pathways were also involved. The previous experiment has shown that concomitant treatment of granulosa cells with melittin plus LHRH does not further increase P_4 production induced by LHRH alone, presumably implying that the effect of LHRH on P_4 production already included the action of endogenous AA (chapter 4). Taken together, P_4 production induced by melittin suggested that the activation of PLA_2, an enzyme that cleaves AA from the 2-acyl position of phospholipids, participated in controlling P_4 production as well as PGE_2 in granulosa cells. More importantly, the present data clearly demonstrated that treatment of granulosa cells with AA for 5h enhanced P_4 production (Fig. 41). This stimulation was dose dependent, within a rather narrow range (i.e. between $3x10^{-7}M$ to $10^{-5}M$) (Fig. 42). The use of the exogenous AA was closely related to the physiologic situation since it was converted to ovarian cyclooxygenase or lipoxygenase metabolites at the site where the appropriate biosynthetic process was present. Both intracellular P_4 concentration and the accumulation of P_4 in the culture medium were increased in the presence of AA. Cell viability was not affected by these dosages of AA, as judged by trypan blue exclusion. In this regard, similar doses of AA have recently been reported to stimulate hormone production in other endocrine tissues. For example, in anterior pituitary cells, AA at $5x10^{-5}M$ or $10^{-4}M$ was a potent secretagogue for LH release (Chang et al., 1986); at $10^{-5}M$ and $10^{-4}M$, AA stimulated ACTH release (Abou-Samra et al., 1986). PRL secretion from GH_3

cells was stimulated by AA at $3x10^{-6}M$ (Kolesnick et al., 1984). Likewise, AA at 10^{-5} to $10^{-6}M$ resulted in the increase of oxytocin in corpus luteam (Hirst et al., 1988), and AA at $10^{-7}M$ to $10^{-5}M$ enhanced testosterone production by Leydig cells (Lin, 1985).

In the present study, the effect of AA on P_4 production was found to be similar to that of LHRH, although the stimulation of P_4 due to AA was somewhat slower in onset compared with that due to LHRH. P_4 levels in the culture medium were identical 5h after the addition of LHRH or AA (Fig. 45). When present together, the effects of AA and LHRH or LHRH agonist became additive; this could be seen as early as 3h after treatment. Since AA also greatly potentiated the stimulation of P_4 production by TPA, the synergistic effect of AA on LHRH-induced P_4 production perhaps reflected a potentiation by AA on LHRH-induced PKC activity (Fig. 44-47). The relatively higher dose and longer time required for the action of AA on P_4 production may be due to the rate of penetration of AA into the cell membrane and the conversion of AA to its metabolites. Recently, PKC activity has been characterized in ovarian tissues (Noland and Dimino, 1986; Davis and Clark, 1983). The highest specific activities were found in cytosol, followed by microsomes and mitochondria (Noland and Dimino, 1986). Several laboratories have reported that activation of PKC by phorbol esters, such as TPA, stimulates basal P_4 production in rat granulosa cells (Wang and Leung, 1987; Kawai and Clark, 1985; Shinohara et al., 1986).

Thus, the present results could be taken to suggest that the stimulatory action of LHRH on ovarian steroidogenesis is mediated, in part at least, by PKC and potentiated by LHRH-induced AA release. Phosphorylation of cytochrome P-450, which is responsible for cholesterol side-chain cleavage, may also result from activation of PKC in steroidogenic tissues (Vilgrain et al., 1984). It seems plausible that LHRH-induced AA could play a second messenger role by amplification of PKC activity, as has been proposed recently for other signalling systems (McPhail et al., 1984; Murakami and Routtenberg, 1985). In support of this theory, it has recently been documented that AA and PKC synergistically mediate the stimulation of gonadotrophin secretion by LHRH in anterior pituitary cells (Chang et al., 1986).

In the same experiment, the effects of other unsaturated fatty acids on P_4 production were also examined (Fig. 43). All the treatments were at the dose of 10^{-5}M which was the maximal effective concentration for AA on stimulating the production of P_4. 11,14 eicosadenoic acid (C18:2), linoleic acid (C18:2), gamma-linolenic acid (C18:3), homo-gamma-linolenic acid (C18:3) like AA, could increase P_4 production in 5h cell incubation. Fatty acids stimulated P_4 production in a order of AA> homo-gamma-linolenic> gamma-linolenic> 11,14 eicosadienoic acid. Another unsaturated fatty acid, oleic acid (C18:1), failed to stimulate P_4 production. These data were similar to the effects of AA and other unsaturated fatty acids on PRL secretion in human decidual tissue (Handwerger et al 1981).

The mechanism of these unsaturated fatty acids on P_4 production was not clear. However, these unsaturated fatty acids are used for cell structures and can convert to AA cascade which could naturally exist in the cell membrane, or produce other series of PGs and LTs (Crawford, 1983). Therefore, a large membrane source of PGs and LTs precursor was provided.

The mechanism by which AA stimulates P_4 production was not known. Previously, LHRH and its agonists have been shown to stimulate PG production in rat granulosa cells (Clark, 1982). The stimulatory effect of LHRH on PG synthesis was additive but apparently distinct from that induced by LH. LH or hCG, as well, has been reported to stimulate ovarian lipoxygenase activity in vivo and in vitro. The activation of lipoxygenase might be correlated with follicular rupture at ovulation (Reich et al., 1983; Reich et al., 1985). In the present study, the possible involvement of PGs was examined using indomethacin that inhibits the cyclooxgenase pathway of AA metabolism. The addition of indomethacin to granulosa cells did not alter LHRH- or AA-induced P_4 production during a 5h culture period (Fig. 48 and 49). Although the blockade of prostaglandin did not affect P_4 production, the inhibition of cyclooxygenase with indomethacin did block ovulation in rat (Amrstrong and Grinwich, 1972), rabbit (Armstrong et al, 1974) and marmoset monkeys (Mai et al., 1975). In addition, in indomethacin-blocked rats, administration of PGE_2 can induce ovulation (Tsafriri et al., 1972). Further, in the absence of prostaglandins, the rupture of ovarian follicle did not occur

(LeMaire and Marsh, 1975). AA can be also converted by the lipoxygenase enzymes to a variety of HPETEs that would be rapidly reduced to their respective HETEs, and 5-HPETE gives rise to another series of products known as the leukotrienes. The contribution of the lipoxygenase pathways to the stimulation of P_4 synthesis was further investigated utilizing NDGA, an effective inhibitor of lipoxygenase pathway of AA in follicular tissue (Reich et al., 1983). AA-induced P_4 production was reduced to the level as that caused by NDGA, and LHRH-induced P_4 formation was only partially suppressed (Fig. 48 and 49). Although NDGA reduced AA- and LHRH-stimulated P_4 production, the basal level of P_4 was increased by NDGA, which might result from the increase in the precursor for PGs synthesis. These results indicated that there may be a stimulatory role for PGs in steroidogenesis. Indeed, exogenous PGE_2 has been shown to stimulate cAMP, estrogen and P_4 production (Richards et al., 1976). Furthermore, the partially inhibitory effect of NDGA on LHRH-induced P_4 production supports the notion that multiple second messengers were involved in the action of LHRH. Besides the release of AA from plasma membrane, LHRH also induced the formation of DG which leads the activation of PKC, and IP_3 which causes Ca^{2+} mobilization. These different pathways cooperated each other and thus contributed to the action of LHRH. The finding that LHRH- or AA-stimulated P_4 production was not influenced by the presence of indomethacin supported the previous observations that LH induced P_4 production was not affected by the

indomethacin-blocked PG formation (Clark, 1982), and that the enhancement of FSH or CT-induced PGE_2 production by LHRH or TPA did not tightly couple to the production of progesterone (Chapter 4). Taken together, LHRH-stimulated P_4 production, partially by increasing free AA and by converting AA to its metabolites, did not result from PG formation. Recently, AA has been implicated in the secretion of oxytocin in ovine corpus luteum. The results showed that PGE_2 and PGF_{2alpha} do not stimulate oxytocin secretion, and AA may have its effect via the lipoxygenase pathway (Hirst et al., 1988), further implicating the involvement of lipoxygenase metabolites in regulating ovarian functions.

The inhibitory effect of NDGA on LHRH- or AA-induced P_4 formation indicated that lipoxygenase metabolites of AA had a role in the P_4 production induced by LHRH. This hypothesis was further investigated in the present study using several HETEs and HPETEs from the 5-, 12-, and 15-lipoxygenase metabolism of AA. The results indicated that at least some lipoxygenase metabolites of AA were capable of enhancing the formation of P_4 by rat granulosa cells in a dose dependent manner (Fig. 50). The stimulatory effects of 12-HETE on P_4 production appeared to be more potent than that of 5-HETE, 5-HPETE or 15-HPETE. In addition to P_4, the formation of PGE_2 was also stimulated by several of the AA metabolites (Fig. 51). This effect of AA metabolitws is not due to cross-reaction of the metabolites in the PGE_2 assay, since at the concentrations used the metabolites do not cross-react. At $5x10^{-6}M$, 12-HETE and 15-

HETE were more potent than 5-HETE in this regard. Like AA, 5-HETE, 5-HPETE, 12-HETE, 15-HETE and 15-HPETE increased basal P_4 production and further augmented the LHRH-induced P_4 production. Also, at 10^{-6}M, 12-HETE and 15-HETE stimulated basal PGE_2 formation and potentiated the stimulation of PGE_2 formation induced by LHRH (Fig. 52). Since very similar results were observed with TPA (Fig. 53), the facilitatory effects of HETEs and HPETEs on LHRH-induced P_4 and PGE_2 production may be due to the interaction of these AA metabolites with LHRH-activated PKC. Since the present data showed the metabolites of lipoxygenase pathway of AA-stimulated PGE_2 production, one might speculate that HETEs or HPETEs act as internal regulators between the metabolites of lipoxygenase and cyclooxygenase pathway. Previous studies have shown that both LH and hCG regulate lipoxygenase activity, but the action of LHRH on these enzymes needs further investigation. It has been reported that both 5- and 15-lipoxygenase require calcium for activity in platelet (Pace-Asciak and Smith, 1986), thus, LHRH-induced rapid increase in $[Ca^{2+}]i$ might be related to the activity of these enzymes in the ovarian cells as well.

There was increasing evidence to support the notion that lipoxygenase metabolites of AA were potent mediators of hormone production in different endocrine tissues. One or more of the cyclooxygenated and/or lipoxygenated metabolites of AA might be a component of the cascade of reactions initiated by LHRH and ultimately result in LH secretion in pituitary (Kiesel et al., 1986; Kiesel et al., 1987). It has also been reported that

leukotrienes are effective stimulators of LH release from dispersed rat anterior pituitary cells (Kiesel et al., 1987; Kiesel et al., 1986; Hurling et al., 1985). Lipoxygenase products of AA metabolism have already been shown to stimulate PRL release (Kiesel et al., 1987). Yamamoto et al. have reported that 5-HETE stimulates insulin release in pancreatic islets (Yamamoto et al., 1983). In bovine corpus luteum, 5-HETE reduced the biosynthesis of P_4 and 6-keto-PGF_{1alpha}, while the synthesis of PGF_{2alpha} was unaffected (Milvae et al., 1986). Inhibition of lipoxygenase activity with NDGA, BW 755C and FPL-55712 resulted in partial blockade of ovulation (Reich et al, 1983; Reich et al., 1985). Although high concentration of 5-HETE had been found in bovine luteal tissue, indicating a physiological importance of those compounds as regulator of ovarian functions (Milvae et al., 1986), thus far, there was no evidence to show that the receptors of these hydroperoxy acids exist on rat granulosa cell. Whether HETEs and HPETEs prove to be intracellular rather than extracellular signals remains to be determined. The 5-lipoxygenase pathway is of special interest because 5-HPETE can be rapidly converted to leukotrienes that are presumably the most active of the lipoxygenase metabolites of AA (Samuelsson, 1983; Morris et al., 1982). In view of the present demonstration of stimulatory effects of 5-HPETE on P_4 and PGE_2 production, the role of leukotrienes on ovarian cell function warrants further investigation.

In addition, the stimulatory role of AA was further

examined in the present study. The inhibitory or stimulatory effects of LHRH on P_4 accumulation with or without the presence of FSH during the different culture period (Fig. 54) further confirmed the previous studies that the inhibitory action of LHRH on granulosa cell steroidogenesis was only observed after 24h in the presence of exogenous gonadotropins, or other cAMP stimulating agents, whereas LHRH did not influence steroidogenesis induced by gonadotrophins in short term incubations (Knecht et al., 1982; Hsueh and Schaeffer, 1985; Hillensjo et al., 1982). The reason for the apparent delay in the onset of this inhibitory effect was not known, although it was believed that LHRH-induced membrane polyphosphoinositide breakdown led to the formation of IPs and DG, and the release of AA, might participate in the action of LHRH (Ma and Leung, 1985; Davis et al., 1986; Davis et al., 1987; Minegishi and Leung, 1985; Wang and Leung, 1987). Unlike the markedly inhibitory effect of LHRH and TPA, AA did not affect the magnitude of P_4 production induced by FSH during a 24h incubation period (Fig. 55). Since the effect of TPA was essentially similar to that of LHRH during long term granulosa cell culture, activation of protein kinase C may participate in the inhibitory action of LHRH at two distinct sites, the gonadotrophin receptor/adenylate cyclase complex and a site distal to the generation of cAMP (Welsh et al., 1984; Barry et al., 1985). Likewise, calcium mobilization may also be involved in the action of LHRH to inhibit gonadotrophin induced cAMP and steroid formation in granulosa cells (Ranta et al.,

1983; Leung et al., 1988). On the other hand, the findings with exogenous AA suggested that AA mediated a stimulatory, rather than inhibitory role in the action of LHRH (Wang and Leung, 1988). To further examine this hypothesis, granulosa cells were treated with FSH (with or without LHRH or TPA) for 18h, at which time the inhibitory effect of LHRH or TPA was already evident. Addition of AA during a further 6h incubation partially reversed the inhibitory effect of LHRH or TPA on FSH- or CT-induced P_4 production (Fig. 56-58). AA was also added to some cells which had been pretreated with LHRH or TPA alone (i.e. in the absence of FSH) for 18h, and the response of the cells to AA was quite similar to that of untreated granulosa cells given AA during a 5h incubation (Fig. 59). The previous studies have reported that LHRH and TPA share a similar inhibitory mechanism of action on FSH induced P_4 production, and the inhibitory effects of LHRH or TPA could not be reversed by the addition of FSH (Knecht et al., 1982; Shinohara et al., 1985). The present data suggested that P_4 production elevated by AA was mediated by a mechanism which was not suppressed by LHRH and TPA, and granulosa cells still retained the ability to respond to AA following LHRH or TPA pretreatment.

The effect of AA on the enzymes involved in progesterone synthesis and metabolism was further examined. Activation of the side chain cleavage enzymes (SCC), and 3-beta-HSD/Δ^5-Δ^4-isomerase convert cholesterol to P_4 via pregnenolone. On the other hand, P_4 is converted by 20-alpha-HSD to its inactive form, 20-alpha-hydroxy-pregn-4-en-3-one (20-alpha-OH-P).

Previous studies have indicated that LHRH alone increases 3-beta-HSD activity by an increase in the apparent V_{max} and is accompanied by increased accumulation of pregnenolone, P_4 and 20-alpha-OH-P production (Jones and Hsueh, 1981a; 1982b). Moreover, the inhibitory effect of LHRH on FSH induced 3-beta-HSD activity, FSH induced pregnenolone and P_4 production have also been observed during 24h granulosa cell culture, and this inhibitory action of LHRH is characterized by a decrease in the apparent V_{max} without an alteration of the K_m of the enzyme (Jones and Hsueh, 1981b; 1982a; 1982b). TPA exerted a similar effect on enzyme activities and FSH induced hormone production to LHRH (Jones and Hsueh, 1982a; 1982b; Welsh et al., 1984). TPA alone stimulated P_4 production and the activities of 3-beta-HSD and 20-alpha-HSD, leading to increased production of progestin. In contrast, TPA inhibition of P_4 biosynthesis induced by gonadotrophin was accompanied by reduction of 3-beta-HSD activity. The increase in 20-alpha-HSD activity resulted in the conversion of biological active P_4 to 20-alpha-OH-P, a biological inactive metabolites. The effects of AA, LHRH and/or TPA on P_4 and 20-alpha-OH-P accumulation were compared in the present study (Fig. 60). AA, LHRH, or TPA each stimulated the production of P_4 and AA enhanced the P_4 production induced by LHRH and TPA (Fig. 60; A panel). LHRH or TPA markedly increased 20-alpha-OH-P production, however AA was only marginally effective in increasing 20-alpha-OH-P (Fig. 60; panel B). In addition, AA did not show any synergistic effect with LHRH or TPA on 20-alpha-OH-P production. These results

indicate that AA increases P_4 production by stimulating biosynthesis rather than significantly altering 20-alpha-HSD activity.

To further examine the action of AA on SCC activity, a substrate for the SCC enzymes, 25-OH-cholesterol, has been used to increase P_4 formation. 25-OH-cholesterol is a water soluble steroid which readily enters cells and is metabolized to steroid hormones in mitochondria (Toaff et al., 1982; Lino et al., 1985). Several steps in the cholesterol SCC reaction such as uptake of cholesterol by mitochondria, the intramitochondrial access of cholesterol to the SCC enzyme complexes, and the modulation of the mitochondrial cytochrome P-450 levels, have been suggested to be under hormone control (Leaven and Boyd, 1981; Sulimovici and Boyd, 1968). In the present study, it was observed that both LHRH and TPA enhanced P_4 production in the presence of 25-OH-cholesterol (Fig. 61). Since 3-beta-HSD/ Δ^5- Δ^4-isomerase activity was not rate-limiting in granulosa cells, the increase in progesterone production in the presence of 25-OH-cholesterol most likely reflected the increased availability of substrate to SCC, and the stimulation of P_4 production by 25-OH-cholesterol indicated that the SCC enzymes were substrate limited as previously reported (Bagavandoss and Midgley, 1987; Toaff et al., 1982). Furthermore, AA increased P_4 production, in the presence of 25-OH-cholesterol, but to a lesser extent than that induced by LHRH or TPA (Fig. 61), suggesting that the AA stimulation of P_4 also takes place at the level of SCC which enhanced substrate

uptake by mitochondria. Interestingly, AA failed to further enhance P_4 production in the presence of cholesterol substrate. The combined treatment of granulosa cells with AA plus LHRH or with AA plus TPA apparently caused maximal activity of the SCC enzymes; therefore addition of 25-OH-cholesterol failed to further enhance P_4 production.

In addition, the in vivo synthesis of ovarian pregnenolone is from cholesterol that is taken up from the plasma, liberated from cholesterol ester stored within cytoplasmic lipid droplets and synthesized in the ovarian cell from 2 carbon components. As demonstrated in granulosa cells cultured in serum free medium, cholesterol could come from de novo biosynthesis, which is dependent on the activities of the rate limiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (Dorrington and Armstrong, 1979; Wang and Hsueh, 1979). AA may also increase enzyme activity in some steps prior to pregnenolone synthesis. It is possible that AA-induced P_4 production is due to either the increased endogenous synthesis of cholesterol, or the liberation of cholesterol from cholesterol esters. There would also be a combination of the above reactions in response to AA. However, any of these mechanisms could account for the observed increase in AA-induced P_4 production.

It is of interest that the inhibitory effect of LHRH on FSH induced ovarian steroid hormone production was only observed after a relatively long time in culture (Fig. 54). One of the proposed mechanisms was that LHRH further enhanced

gonadotrophin induced 20-alpha-HSD activity by which LHRH diminished the gonadotrophin stimulation of P_4 production. The LHRH stimulation of 20-alpha-HSD in gonadotrophin treated cells was the result of changes in enzyme activity, rather than enzyme affinity for the substrate (Phillip et al., 1980). Assuming that the calcium and PKC pathways can partially mediate the inhibitory action of LHRH on granulosa cells, it can be postulated that LHRH-induced liberation of AA may somehow antagonize the inhibitory component of LHRH action. This was suggested by the present findings that AA did not decrease FSH induced P_4 accumulation even after 24h (Fig. 53) and that acute addition of AA to the FSH and CT-pretreated cells caused a partial reversal of the inhibitory effect of TPA or LHRH on P_4 production (Fig. 56-58). Moreover, AA mainly stimulated P_4 production, but TPA stimulated both P_4 and 20a-OH-P effectively (Fig. 60). These data suggest that activation of PKC may well mediate the long term inhibitory action of LHRH on P_4 accumulation, by converting P_4 to 20-alpha-OH-P. In contrast, AA (or its active metabolites) most likely played a role in the short term stimulatory effect of LHRH on P_4 production by enhancing the activity of SCC. Taken together, the potential inhibitory effects of LHRH (via activation of PKC) might have been prevented by AA during the 5h incubations. After that, the LHRH-induced free AA may convert to some inactive metabolites and the inhibitory component of LHRH action became dominant.

In addition, study of the functions of TPA, A23187 and AA

has provided evidence for a participation of PKC, Ca^{2+} and metabolites of AA in the mediation of LHRH action (chapter 4). However, it was unlikely that cellular responses involving PKC, calcium and metabolites of AA were just limited to the cell membrane and cytoplasm. There was accumulating evidence that extranuclear events were coordinated by nuclear components, including changes in specific gene expression, i.e. the regulation of $P450_{SCC}$ mRNA by FSH (Richards and Hedin, 1988). Therefore, the action of LHRH on FSH induced ovarian steroidogenesis in long term culture may be due to the interaction of these hormones on gene expression. It has already been shown that FSH administration to hypophysectomized rats causes the increase in cytochrome $P450_{SCC}$ mRNA in granulosa cells and FSH induced gene expression which is only clearly demonstrable after 7 to 10h. Thus far, no discussion of LHRH regulation of gene expression has been made in granulosa cells.

In conclusion, the present results strongly support the hypothesis that AA and its lipoxygenase metabolites partially mediate the action of LHRH by playing a stimulatory role in the direct effects of LHRH on ovarian hormone production. Furthermore, it indicates that the actions of LHRH or LHRH-like peptide on granulosa cells are mediated by the different intracellular signal pathways, and that the complex interplay between these pathways ultimately dictates the time-dependent steroidogenic response of the ovary to LHRH or LHRH-like peptide.

General Summary

Although gonadotropins are the major trophic hormones that regulate ovarian functions, increasing evidence suggests that local regulators participate in paracrine or autocrine control of ovarian functions.

The direct actions of LHRH on rat ovarian cells have been documented. Unlike gonadotropins, LHRH does not use cAMP as its second messenger. Increasing evidence shows that the initial action of LHRH involves a rapid alteration in the metabolism of membrane inositol lipids in the ovary. In the present study, the actions of LHRH on the breakdown of membrane phosphoinositides, changes of intracellular Ca^{2+}, production of steroid hormones and prostaglandins in rat granulosa cells were extensively studied. In radiolabeled rat granulosa cells, the rapid and specific formation of inositol 1,4,5-trisphosphate and diacylglycerol, and the release of arachidonic acid were observed shortly after addition of LHRH. LHRH also caused a rapid and transient increase in $[Ca^{2+}]i$ in the majority of granulosa cells as assessed by fura-2 microspectrofluorimetry. Inositol 1,4,5-trisphosphate, which is produced simultaneously with diacylglycerol by PLC hydrolysis of PIP_2, may be responsible for the LHRH induced rapid and transient alterations of $[Ca^{2+}]i$ in granulosa cells. It is known that LHRH exerts either stimulatory or inhibitory actions on ovarian cells depending on the culture period, the presence of other hormones such as gonadotropins, and the

nature of the hormone examined. To test the hypothesis that the effects of LHRH on granulosa cells were mediated, at least in part, by calcium and PKC, the effects of the calcium ionophore A23187 and the phorbol ester TPA on the production of progesterone and PGE_2 have been examined. The present study demonstrated that LHRH inhibited the production of progesterone stimulated by FSH, while simultaneously enhancing PGE_2 production stimulated by FSH. These data suggest that the action of LHRH is mainly at a step(s) following gonadotropin induced cAMP formation. TPA and A23187 can mimic the actions of LHRH. Interestingly, TPA acted synergisticly with A23187 on PGE_2 production but not on the production of progesterone, suggesting multiple aspects of PKC and Ca^{2+} action on granulosa cells. It appears that activation of PKC and alteration of $[Ca^{2+}]i$ not only mediates cellular processes, but also alters membrane phosphoinositide metabolism, thus providing a potential feedback control mechanism. Increased free arachidonic acid level induced by LHRH serves as the precursor for the synthesis of cyclooxygenase and lipoxygenase metabolites of arachidonic acid. Prostaglandins are the cyclooxygenase metabolites of arachidonic acid which play a very important role in reproductive functions of the ovary. Although the production of prostaglandin may not be tightly coupled to progesterone production, it is certainly involved in the ovulation process. In the present study, lipoxygenase pathway metabolites apparently participated in the stimulatory action of LHRH probably by enhancing the action of protein

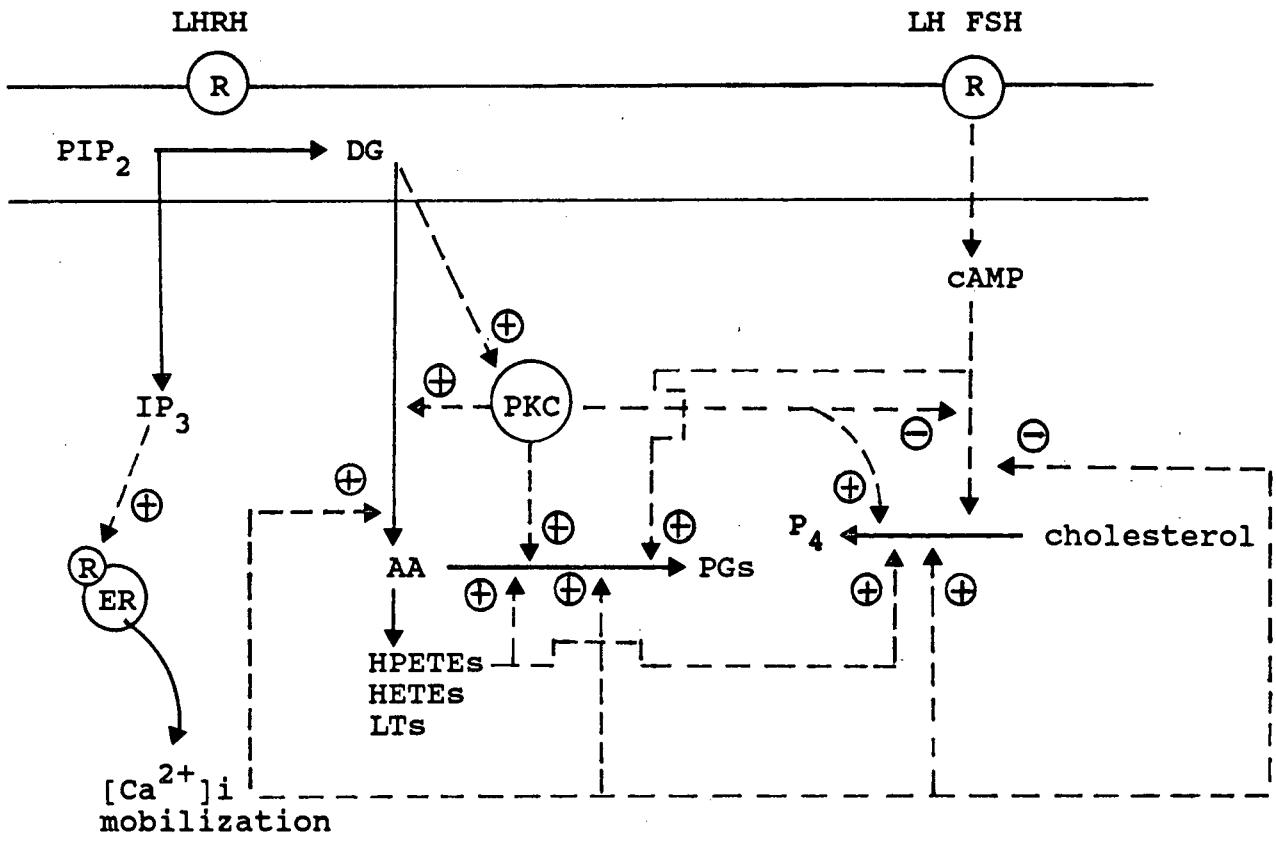

Fig. 62. Illustration of the interactions between luteinizing hormone-releasing hormone (LHRH) and gonadotrophin second messenger pathways. Abbreviations: LH, luteinizing hormone; FSH, follicle stimulating hormone; R, receptor; DG, 1,2-diacylglycerol; cAMP, 3'5'-cyclic adenosine monophosphate; PKC, protein kinase C; ER, endoplasmic reticulum; AA, arachidonic acid; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; PG, prostaglandin; $[Ca^{2+}]i$, intracellular calcium ion concentration; IP_3, inositol 1,4,5,-trisphosphate; P_4, progesterone.

kinase C on the enzymes involved in steroidogenesis. The interaction between the gonadotropins and LHRH are summarized schematically in Fig. 62.

The evidence for the possible paracrine or autocrine roles of LHRH in ovarian cells is strengthened by the demonstration of the presence of LHRH-like peptides in human, rat, bovine and ovine ovaries in other sdudies. The involvement of these different hormonal systems and multiple second messenger mechanisms in the regulation of granulosa cell function ensures the optimal ovarian hormone synthesis and the growth of the ovarian follicles. In addition, rat granulosa cells serve as an ideal model for studies on the mechanism of hormone action because of the presence of both cAMP and Ca^{2+}-protein kinase C pathways. The present <u>in vitro</u> findings should help future elucidation of the processes of ovarian hormone production and ovulation.

References

Aberdam, E. and Dekel, N. 1985 Activators of protein kinase C stimulate meiotic maturation of rat oocytes. Biochem Biophys Res Commun 132:570-574

Abou-samra, A.B., Catt, K.J. and Aguilera, G. 1986 Role of arachidonic acid in the regulation of adenocorticotropin release from rat anterior pituitary cell cultures. Endocrinology 119:1427-1431

Adashi, E.Y. and Hsueh, A.J.W. 1982 Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells. J Biol Chem 257:6077-6083

Adashi, E.Y., Resnick, C.E., D'Ercole, A.J., Svoboda, M.E. and Van Wyk, J.J. 1985 Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. Endocr Rve 6:400-420

Aloyo, V.J., Zwiers, H. and Gispen, W.H. 1983 Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent protein kinase and B-50 protein kinase. J Neurochem 41:649-653

Amsterdam, A., Nimord, A., Lamprecht, S.A. and Burstein Linder, H.R. 1979 Internalization and degradation of receptor-bound hCG in granulosa cell culture. Am J Physiol 236:E129-138

Amsterdam, A. and Rotmensch, S. 1987 Structure-function relationships during granulosa cell differentiation. Endocr Rev 8:309-336

Arimura, A., Serafini, P., Talbot, S. and Schally, A.V. 1979 Reduction of testicular luteinizing hormone/human chorionic gonadotropin receptors by [D-Trp 6]-luteinizing hormone releasing hormone in hypophysectomized rats. Biochem Biophys Res Commun 90:687-693

Armstrong, D.T., Lee, T.P. and Miller, L.S. 1970 Stimulation of progesterone biosynthesis in bovine corpora lutea by luteinizing hormone in the presence of an inhibitor of cholesterol synthesis. Biol Reprod 2:29-36

Armstrong, D.T., Knudsen, K.A. and Miller, L.S. 1970 Effect of prolactin upon cholesterol metabolism and progesterone biosynthesis in corpora lutea of hypophysectomized rats during pseudopregnancy. Endocrinology 86:634-641

Armstrong, D.T. and Grinwich, D.L. 1972 Blockade of spontaneous and LH-induced ovulation in rats by indomethacin, an inhibitor of prostaglandin biosynthesis. Prostaglandins 1:21-28

Armstrong, D.T., Grinwich, D.L., Moon, Y.S. and Zanecnik, J. 1974 Inhibition of ovulation in rabbit by intrafollicular injection of indomethacin and PGF_{2alpha} anti-serum. Life Sci 14:129-140

Asch, R.H., Sickle, M.V., Rettori, V., Balmaceda, J.P., Eddy, C.P., Coy, D.H. and Schally, A.V. 1981 Absence of LHRH binding sites in corpora lutea from rhesus monkeys (Macaca mulatta). J Clin Endocrinol Metab 53:215-217

Asem, E.K., Molnar, M. and Hertelendy, F. 1987 Luteinizing hormone-induced intracellular calcium mobilization in granulosa cells: Comparison with forskolin and 8-bromo-adenosine 3',5'-monophosphate. Endocrinology 120:853-859

Aten, R.F., Williams, A.T. and Behrman, H.R. 1986 Ovarian gonadotropin-releasing hormone-like protein (s): demonstrations and characterization. Endocrinology 118:961-967

Aten, R.F., Ireland, J.J., Weems, C. W. and Behrman, H.R. 1987 Presence of gonadotropin-releasing hormone-like protein in bovine and ovine ovaries. Endocrinology 120:1727-1733

Aub, D.L., McKinney, J.S. and Putney, J.W., Jr. 1982 Nature of the receptor-regulated calcium pool in the rat parotid gland. J Physiol 331:557-565

Bagavandoss, P. and Midgley, A.R., Jr. 1987 Lack of difference between retinoic acid and retinal in stimulating progesterone production by luteinizing granulosa cells in vitro. Endocrinology 121:420-428

Bahr, J., Kao, L. and Nalbandov, A.V. 1974 The role of catecholamines and nerves in ovulation. Biol Reprod 10:273-290

Baum, M.S. and Rosberg, S. 1987 A phorbol ester, phorbol 12-myristate 13-acetate, and a calcium ionophore, A23187, can mimic the luteolytic effect of prostaglandin F_{2alpha} in isolated rat luteal cells. Endocrinology 120:1019-1026

Beers, M.H., Strickland, S. and Reich, E. 1975 Ovarian plasminogen activator: Relationship to ovulation and hormonal regulation. Cell 6:387-394

Behrman, H.R. 1979 Prostaglandins in hypothalamo-pituitary and ovarian function. Ann Rev Physiol 41:685-700

Bell, R.L. and Majerus, P.W. 1979 Thrombin-induced hydrolysis of phosphatidylinositol in human platelets. J Biol Chem 255:1790-1792

Bell, R.L., Kennerly, D.A., Stanford, N. and Majerus, P.W. 1979 Diglyceride lipase: a pathway for arachidonate release from

human platelets. Proc Natl Acad Sci USA 76:3238-3241

Ben-Ze'ev, A. and Amsterdam, A. 1987 In vitro regulation of granulosa cell differentiation. J Biol Chem 262:5366-5376

Berridge, M.J. 1981 Phosphatidylinositol hydrolysis: a multifunctional transducing mechanism. Mol Cell Endocrinol 24:115-140

Berridge, M.J., Downes, C.P. and Hanley, M.R. 1982 Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem J 206:587-589

Berridge, M.J. 1983 Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. Biochem J 211:849-858

Berridge, M.J. 1984 Inositol triphosphate and diacylglycerol as second messengers. Biochem J 820:345-360

Berridge, M.J. 1987 Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem 56:159-193

Bjersing, L. 1967 Histochemical demonstration of ^{5}-3-beta- and 17-beta-hydroxysteroid dehydrogenase activities in porcine ovary. Histochemie 10:295-304

Bjersing, L. and Carstensen, H. 1967 Biosynthesis of steroids by granulosa cells of the porcine ovary in vitro. J Reprod Fertil 14:101-111

Bone, E.A., Alling, D.W. and Grollman, E.F. 1986 Norepinephrine and thyoroid-stimulating hormone induce inositol phosphate accumulation in FRTL-5 cells. Endocrinology 119:2193-2200

Brodie, A.M., Schwarzel, W.C. and Brodie, H.J. 1976 Studies on the mechanism of estrogen biosynthesis in the rat ovary. J Steroid Biochem 7:787-793

Brown, J.L. and Reeves, J.J. 1983 Absence of specific LHRH receptors in ovine, bovine, and porcine ovaries. Biol Reprod 29:1179-1182

Brown, M.S., Kovanen, P.T. and Goldstein, J.L. 1981 Regulation of plasma cholesterol by lipoprotein receptors. Science 212:628-635

Burgess, G.M., Irvine, R.F., Berridge, M.J., McKinney, J.S. and Putney, J.W. 1984 Actions of inositol phosphates on Ca^{2+} pools in guinea pig hepatocytes. Biochem J 224:741-746

Burgess, G.M., Godfrey, P.P., McKinney, J.S., Berridge, M.J.,

Irvine, R.F. and Putney, J.W., Jr. 1984 The second messenger linking receptor activation to internal Ca^{2+} release in liver. Nature 309:63-66

Byskov, A.G. and Rasmussen, G. 1973 Ultrastructure studies of the developing follicle. In: The Development and Maturaion of the Ovary and Its Function, International Congress Steries No. 267 edited by H. Peters. pp. 55-62

Byskov, A.G. 1978 The anatomy and ultrastructure of the rete system in the fetal mouse ovary. Biol Reprod 19:720-735

Carafoli, E. and Crompton, M. 1978 The regulation of intracellular calcium. Current Topics in Membranes and Transport 10:151-216

Carlson, J.C., Nelson, S.E. and Bols, N.C. 1986 Progesterone secretion by primary cultures of rat luteal cells Endocrine Research 12:1-20

Change, J.P., Graeter, J. and Catt, K.J. 1986 Coordinate actions of arachidonic acid and protein kinase C in gonadotropin-releasing hormone-stimulated secretion of luteinizing hormone. Biochem Biophys Res Commun 134:134-139

Charest, R., Blackmore, P.F., Berthon, B. and Exton, H. 1983 Changes in free cytosolic Ca^{2+} in hepatocytes following alpha-adrenergic stimulation. J Biol Chem 258:8769-8773

Cheatum, S.G. and Warren, J.C. 1966 Purification and properties for 3-beta-hydroxysteroid dehydrogenase and 3-ketosteroids isomerase from bovine corpora lutea. Biochim Biophys Acta 122:1-6

Clapper, D.A. and Conn, P.M. 1985 GnRH stimulation of pituitary gonadotrope cells produces an increase in intracellular calcium. Biol Reprod 32:269-278

Claret-Berthon, B., Claret, M. and Mazet, J.L. 1977 Fluxes and distribution of calcium in rat liver cells: Kinetic analysis and identification of pools. J Physiol 272:529-552

Clark, M.R. 1982 Stimulation of progesterone and prostaglandin E2 accumulation by luteinizing hormone-releasing hormone (LHRH) and LHRH analogs in rat granulosa cells. Endocrinology 110:146-152

Clark, M.R., Marsh, J.M. and LeMaire, W.J. 1978 Mechanism of luteinizing hormone regulation of prostaglandin synthesis in rat granulosa cells. J Biol Chem 253:7757-7761

Clark, M.R., Thibier, C., Marsh, J.M. and LeMaire, M. 1980 Stimulation of prostaglandin accumulation by luteinizing

hormone-releasing hormone (LHRH) and LHRH analogs in rat granulosa cells in vitro. Endocrinology 107:17-23

Clayton, R.N., Harwood, J.P. and Catt, K.J. 1979 Gonadotropin-releasing hormone analogue binds to luteal cells and inhibits progesterone production. Nature 282:90-92

Clayton, R.N. and Catt, K.J. 1981 Gonadotropin-releasing hormone receptors: characterization, physiological regulation, and relationship to reproductive function. Endocrine Rev 2:186-209

Clayton, R.N. and Huhtaniemi, I.T. 1982 Absence of gonadotropin-releasing hormone receptors in human gonadal tissue. Nature 299:56-59

Condon, W.A. and Black, D.L 1976 Catecholamine-induced stimulation of progesterone by the bovine corpus luteum in vitro. Biol Reprod 15:573-578

Conn, P.M., Ganong, B.R., Ebeling, J., Staley, D., Neidel, J.E. and Bell, R.M. 1985 Diacylglycerols release LH: Structure-activity relations and protein kinase C. Biochem Biophys Res Comm 126:532-539

Conn, P.M., McArdle, C., Andrews, W.V. and Huckle, W. 1987 The molecular basis of gonadotropin-releasing hormone (GnRH) action in the pituitary gonadotrope. Biol Reprod 36:17-35

Cooper, R.H., Coll, K.E. and Williamson, J.R. 1985 Differential effects of phorbol ester on phenylephrine and vasopressin-induced Ca++ mobilization in isolated hepatocytes. J Biol Chem 260:3281-3288

Corvera, S., Schwarz, K.R., Graham, R.M. and Garcia-Sainz, J.A. 1986 Phorbol ester inhibits alpha 1-adrenergic effects and decrease the affinity of liver cell alpha 1-adrenergic receptors for (-)-epinephrine. J Biol Chem 261:520-526

Crawford, M.A. 1983 Essential fatty acids and their prostanoid derivatives. Br Med Bull 39:210-213

Culler, M.D., Tarlatzis, B.C., Lightman, A., Fernandez, L.A., De-Cherney, A.H., Negro-Vilar, A. and Naftolin, F. 1986 Angiotensin II-like immunoreactivity in human follicular fluid. J Clin Endocrinol Metab 62:613-615

Daniel, S.A.J. and Armstrong, D.T. 1980 Enhancement of follicle-stimulating hormone-induced aromatase activity by androgens in cultured rat granulosa cells. Endocrinology 107:1027-1033

Darbon, J.M., Knecht, M., Ranta, R., Dufau, M. and Catt, K.J.

1984 Hormonal regulation of cyclic AMP-dependent protein kinase in cultured ovarian granulosa cells. J Biol Chem 259:14778-14782

Davidson, J., King, J.A. and Millar, R.P. 1987 Luteinizing hormone release from chicken pituitary cells: synergism between calcium and protein kinase C and its inhibition by calmodulin antagonists. Endocrinology 120:692-699

Davis, J.S. and Clark, M.R. 1983 Activation of protein kinase C in the bovine corpus luteum by phospholipids and Ca2+. Biochem J 214:569-574

Davis, J.S., Farese, R.V. and Clark, M.R. 1983 Gonadotropin-releasing hormone (GnRH) stimulates phosphatidylinositol metabolism in rat granulosa cells: mechanism of action of GnRH. Proc Natl Acad Sci USA 80:2049-2053

Davis, J.S., Weakland, L.L., West, L.A. and Farese, R.V. 1986 Luteinizing hormone stimulates the formation of inositol triphosphate and cyclic AMP in rat granulosa cells: evidence for phospholipase C generated second messengers in the action of luteinizing hormone. Biochem J 238:597-604

Davis, J.S., West, L.A. and Farese, R.V. 1986 Gonadotropin-releasing hormone (GnRH) rapidly stimulates the formation of inositol phosphates and diacylglycerol in rat granulosa cells: further evidence for the involvement of Ca++ and PKC in the action of GnRH. Endocrinology 118:2561-2571

Davis, J.S., Weakland, L.L., Farese, R.V. and West, L.A. 1987 Luteinizing hormone increases inositol triphosphate and cytosolic free Ca2+ in isolated bovine luteal cells. J Biol Chem 262:8515-8521

De Chaffoy de Courcelles , D., Roevens, P. and Van Belle, H. 1984 1-oleoyl-2-acetyl-glycerol (OAG) stimulates the formation of phosphatidylinositol 4-phosphate in intact human platelets. Biochem Biophys Res Commun 123:589-595

Dekel, N. and Beers, W.H. 1978 Rat oocyte maturation in vitro: relief of cyclic AMP inhibitions by gonadotropins. Cell Biol 75:4369-4373

Dekel, N. and Aberdam, E. 1985 Activators of protein kinase C stimulate meiotic maturation of rat oocytes. Biochem Biophys Res Commun 132:570-574

Dekel, N., Lawrence, T.S., Gilula, N.B. and Beers, W.H. 1981 Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH. Dev Biol 86:356-362

Dekel, N., Lewysohn, O., Ayalon, D. and Hazum, E. 1988 Receptors for gonadotropin releasing hormone are present in rat oocytes. Endocrinology 123:1205-1207

Dekel, N., Sherizly, I., Tsafriri, A. and Naor, Z. 1982 A comparative study of the mechanism of action of LH and a GnRH analog on the ovary. Biol Reprod 28:161-166

Dekel, N., Sherizly, I., Tsafriri, A. and Naor, Z. 1983 A comparative study of the mechanism of action of LH and a GnRH analog on the ovary. Biol Reprod 28:161-166

Delbeke, D., Scammell, J.G. and Dannies, P.S. 1984 Differences in calcium requirements for forskolin-induced release of prolactin from normal pituitary cells and GH4C1 cells in culture. Endocrinology 114:1433-1400

Dennefors, B.L., Janson, P., Knutson, F. and Hamberger, L. 1980 Steroid production and responsiveness to gonadotropin in isolated stromal tissue of human nenopausal ovaries. Am J Obstet Gynecol 136:997-1002

Dexter, R.N., Fishman, L.M., Ney, R.L. and Liddle, G.W. 1967 Inhibition of adrenal corticosteroid synthesis by aminoglutethimide: studies on the mechanism of action. J Clin Endocrinol Metab 27:473-480

Diaz-Infante, A., Jr., Wright, K.H. and Wallach, E.E. 1974 Effects of indomethacin and prostaglandin F_{2alpha} on ovulation and ovarian contractility in the rabbit. Prostaglandins 5:567-579

Dimino, M.J. and Campbell, M.D. 1976 Progesterone synthesis by luteal mitochondria in vitro. Pro Soc Exp Biol Med 152:54-56

Dixon, J.F. and Hokin, L.E. 1984 Secretagogue-stimulated phosphatidylinositol breakdown in the exocrine pancreas liberates arachidonic acid, stearic acid, and glycerol by sequential actions of phospholipase C and diglycerol lipase. J Biol Chem 259:14418-14425

Dorflinger, L.J., Albert, P.J., William, A.T. and Behrman, H.R. 1984 Calcium is an inhibitor of luteinizing hormone-sensitive adenylate cyclase in the luteal cell. Endocrinology 114:1208-1215

Dorrington, J.H. and Armstrong, D.T. 1979 Effect of FSH on gonadal functions. Recent Prog Horm Res 35:301-342

Dorrington, J., Mckeracher, H., Munshi, S. and Gore-langton, R. 1982 LHRH independently stimulates steroidogenic enzymes in granulosa cell culture. Endocrinology 110 (Suppl):178

Downs, S.M. and Longo, F.J. 1982 Effects of indomethacin on preovulatory follicles in immature superovulated mice. Am J Anat 164: 265-274

Downs, S.M. and Longo, F.J. 1983 An ultrastructural study of preovulatory apical development in mouse ovarian follicles: Effects of indomethacin. Anat Rec 205: 159-168

Dunaif, A.E., Zimmerman, E.A., Friesen, H.G. and Frantz, A.G. 1982 Intracellular localization of prolactin receptor and prolactin in the rat ovary by immunocytochemistry. Endocrinology 110:1465-1471

Eckstein, N., Eshel, A., Eli, Y., Ayalon, D. and Naor, Z. 1986 Calcium-dependent actions of gonadotropin-releasing hormone agonist and luteinizing hormone upon cyclic AMP and progesterone production in rat ovarian granulosa cells Molecular and Cellular Endocrinology 47:91-98

Ekholm, C., Clark, M.R., Magnusson, C., Isaksson, O. and LeMaire, W.J. 1982 Ovulation induction by a GnRH analog in hypophysectomized rats involves prostaglandins. Endocrinology 110:288-290

Enyedi, A., Farago, Sarkadi, B. and Gardos, G. 1984 Cyclic AMP-dependent protein kinase and Ca^{2+}-calmodulin stimulate the formation of polyphosphoinositides in a sarcoplasmic reticulum preparation of rabbit heart. FEBS Lett 176:235-238

Erdo, S.L. and Lapis, E. 1982 Bicuculline-sensitive GABA receptors in rat ovary. Eur J Pharmacol 85:243-246

Espey, L.L. 1980 Ovulation as an inflammatory reaction: a hypothesis. Biol Reprod 22:73-106

Erickson G.F. 1978 Normal ovarian function. Clin Obstet Gynecol 21:31-42

Fanjul, L.F., Ruiz de Galarreta, C.M. and Hsueh, A.J.W. 1983 Progestin augmentation of gonadotropin-stimulated progesterone production by cultured rat granulosa cells. Endocrinology 112:405-407

Farkas, G., Enyedi, A., Sarkadi, B. Gardos, G., Nagy, Z. and Farago, A. 1984 Cyclic AMP-dependent protein kinase stimulates the phosphorylation of phosphatidylinositol to phosphatidylinositol 4-phosphate in a plasma membrane preparation from pig granulocytes. Biochem Biophys Res Commun 124:871-876

Farkash, Y., Timberg, R. and Orly, J. 1986 Preparation of antiserum to rat cytochrome P-450 cholesterol side chain cleavage, and its use for ultrastructural localization of

immunoreactive enzyme by protein A-gold technique. Endocrinology 118:1353-1365

Farookhi, R. 1980 Effects of androgen on induction of gonadotropin receptors and gonadotropin-stimulated adenosine 3',5'-monophosphate production in rat ovarian granulosa cells. Endocrinology 106:1216-1223

Flower, R.J. and Vane, J.R. 1974 Some pharmacologic and biochemical aspects of prostaglandin biosynthesis and its inhibition, In: Robinson, H.J. and Vane, J.R. (eds), Prostaglandin Synthesis Inhibitors. Raven Press, New York, pp. 9-18

Folch, J., Lees, M. and Sloane-Stanley, G.H. 1957 A simple method for the isolation and purification of total lipids from animal tissue. J Biol Chem 226:497-509

Forder, J., Scriabine, A. and Rasmussen, H. 1985 Plasma membrane calcium flux, protein kinase C activation, and smooth muscle contraction. J Pharmacol Exp Ther 235:267-273

Forkert, V.W., Yunis, M. and Schlondorff, D. 1984 Prostaglandin synthesis linked to phosphatidylinositol turnover in isolated rat glomeruli. Biochem Biophys Acta 794:206-217

Fortune, J.E. and Armstrong, D.T. 1977 Androgen production by theca and granulosa isolated from proestrous rat follicles. Endocrinology 100:1341-1347

Fowler, R.E., Fox, N.L., Edwards, R.G., Walters, D.E. and Steptoe, P.C. 1978 Steroidogenesis by cultured granulosa cells aspirated from human follicles using pregnenolone and andrgen as precursors. J Endocrinol 77:171-183

Fukuda, S., Terakawa, N., Sato, B., Imori, T. and Matsumoto, K. 1979 Hormonal regulation of activities of 17beta-dehydrogenases, aromatase and 4-ene-5alpha-reductase in immature ovaries. J Steroid Biochem 11:1421-1427

Garrison, J.C., Johnsen, D.E. and Campanile, C.P. 1984 Evidence for the role of phosphorylase kinase, protein kinase C, and other Ca^{2+}-sensitive protein kinases in the response of hepatocytes to angiotensin II and vasopressin. J Biol Chem 259:3283-3297

Geison, R.L., Banschbach, M.W., Sabeghian, K. and Hokin-Neaverson, M. 1976 Acetylcholine stimulation of selective increase in stearic acid and arachidonic acids in phosphatidic acid in mouse pancreas. Biochem Biophys Res Comm 68:343-349

Gick, G.G. and Bancroft, C. 1985 Regulation by calcium of prolactin and growth hormone mRNA sequences in primary cultures of rat pituitary cells. J Biol Chem 259:10675-10681

Gilman, A.G. 1984 Guanine nucleotide-binding regulatory proteins and dual control of adenylate cyclase. J Clin Invest 73:1-3

Gilman, A.G. 1987 G proteins:transducters of receptor generated signals. Annu Rev Biochem 56:615-649

Goff, A.K. and Armstrong, D.T. 1977 Stimulatory action of gonadotropins and prostaglandins on adenosine 3',5'-monophosphate production by isolated rat granulosa cells. Endocrinology 101:1461-1467

Goff, A.K. and Henderson, K.M. 1979 Changes in follicular fluid and serum concentrations of steroids in PMSG treated immature rats following LH administration. Biol Reprod 20:1153-1157

Goff, A.K., Leung, P.C.K. and Armstrong, D.T. 1979 Stimulatory effect of follicle-stimulating hormone and androgens on the responsiveness of rat granulosa cells to gonadotropins in vitro. Endocrinology 104:1124-1129

Goldring, N.B. and Orly, G. 1985 Concerted metabolism of steroid hormone produced by co-cultured ovarian cell types. J Biol Chem 260:913-920

Goodman, A. and Hodgen, G. 1979 Between-ovary interaction in the regulation of follicle growth: Corpus luteum function and gonadotropin secretion in the primate ovarian cycle. Endocrinology 104:1310-1316

Gore-Langton, R.E., Lacroix, M. and Dorrington, J.H. 1981 Differential effects of luteinizing hormone-releasing hormone on follicle-stimulating hormone-dependent responses in rat granulosa cells and sertoli cells in vitro. Endocrinology 108:812-818

Gospodarowicz, D., III, C.R. and Birdwell, C.R. 1977 Effect of fibroblast and epidermal growth factors on ovarian cell proliferation in vitro. I. Characterization of the response of granulosa cells for FGF and EGF. Endocrinology 100:1108-1120

Gospodarowicz, D., III, C.R. and Birdwell, C.R. 1977 Effect of fibroblast and epidermal growth factors on ovarian cell proliferation in vitro. II. Proliferative response of luteal cells to FGF but not EGF. Endocrinology 100:1121-1128

Gospodarowicz, D. and Bialecki, I. 1979 Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine and human origin. Endocrinology 104:757-764

Greenwald, G.S. 1974 Gonadotropin regulation of follicular development. In: Gonadotropins and Gonadal Function, edited by

N.R. Moudgal. pp. 205-212

Grynkiewicz, G., Poenie, M. and Tsien, Y. 1985 A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 260:3440-3450

Gwynne, J.T. and Strauss III, J.F. 1982 The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocr Rev 3:299-329

Habermann, E. 1972 Bee and wasp venoms. Science 177:314-322

Haksar, A. and Romanoff, E.B. 1971 Electron transport and progesterone biosynthesis in the bovine corpus luteum homogenate. Biochem Biophys Res Commun 43:246-251

Hamberger, L., Hillensjo, T. and Ahren, K. 1978 Steroidogenesis in isolated cells of preovulatory rat follicles. Endocrinology 103:771-777

Handwerger, S., Barry, S., Barrett, J., Markoff, E., Zeitler, P., Cwikei, B. and Siegel, M. 1981 Inhibition of the synthesis and secretion of decidual prolactin by arachidonic acid. Endocrinology 109:2016-2021

Hansson, A., Serhan, C.N., Haeggstrom, J., Ingelman-Sundberg, M. and Samuelsson, B. 1986 Activation of protein kinase C by lipoxin A and other eicosanoids. Intracellular action of oxygenation products of arachidonic acid. Biochem Biophys Res Commun 134:1215-1222

Harman, S.M., Louvet, J-P. and Ross, G.T. 1975 Interaction of estrogen and gonadotrophins on follicular atresia. Endocrinology 96:1145-1152

Harris, C.E., Staley, D. and Conn, P.M. 1985 Diacylglycerols and protein kinase C: potential amplifying mechanism for Ca-mediated gonadotropin-releasing hormone-stimulated luteinizing hormone release. Molecular Pharmacology 27:532-536

Hazum, E. and Nimrod, A. 1982 Photoaffinity-labeling and fluorescence distribution studies on GnRH receptors in ovarian granulosa cells. Proc Natl Acad Sci USA 79:1747-1750

Hazum, E. and Schvartz, I. 1984 Photoaffinity labeling of gonadotropin releasing hormone receptors in control and desensitized pituitary cells. Biochem Biophys Res Commun 125:532-538

Hedin, L., Gaddy-Kurten, G., Kurten, R., DeWitt, D.L., Smith, W.L. and Richards, J.S. 1987 Prostaglandin endoperoxide (PGH) synthase in rat ovarian follicles: Content, cellular distribution and evidence for hormonal induction preceding

ovulation. Endocrinology 121:722-731

Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. 1984 Isoquinoline-sulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochem 23:5036-5041

Higuchi, T., Kaneko, A. Abel, J.H. and Niswender, G.D. 1976 Relationship between membrane potential and progesterone release in ovine corpora luteal. Endocrinology 99:1023-1039

Hillensjo, T. and LeMaire, W.J. 1980 Gonadotropin-releasing hormone agonist stimulate meiotic maturation of follicle-enclosed rat oocytes in vitro. Nature 287:145-146

Hillensjo, T., LeMaire, W.J., Clark, M.R. and Ahren, K. 1982 Effect of GnRH and GnRH agonists upon accumulation of progesterone, cAMP and prostaglandin in isolated preovulatory rat follicles. Acta Endocrinol Copenh 101:603-610

Hillier, S.G., Zeleznik, A.J., Knazek, R.A. and Ross, G.T. 1980 Hormonal regulation of preovulatory follicle maturation in the rat. J Reprod Fertil 60:219-229

Hirst, J.J., Riie, G.E., Jenkins, G. and Thorburn, G.D. 1988 Control of oxytoxin secretion by ovine corpora lutea: effects of arachidonic acid, phospholipases, and prostaglandins. Endocrinology 122:774-781

Holl, R.W., Thorner, M.O. and Leong, D.A. 1988 Intracellular calcium concentration and growth hormone secretion in individual somatotropes: effects of growth hormone releasing factor (GRF) and somatostatin (SRIF). Endocrinology 122:2927-2932

Holt, J.A., Richards, J.S., Midgley, A.R., Jr. and Reichert L.E., Jr. 1976 Effect of prolactin on LH receptor in rat luteal cells. Endocrinology 98:1005-1013

Hopkins, C.R. and Gregory, H. 1977 Topographical localization of the receptors for luteinizing hormone-releasing hormone on the surface of dissociated pituitary cells. J Cell Biol 75:528-540

Hsueh, A.J.W. and Erickson, G.F. 1979 Extrapituitary action of gonadotropin-releasing hormone: direct inhibition of ovarian steroidogenesis. Science 204:854-855

Hsueh, A.J.W. and Ling, N.C. 1979 Effect of an antagonistic analog of gonadotropin-releasing hormone upon ovarian granulosa cell function. Life Sci 25:1223-1230

Hsueh, A.J.W., Wang, C. and Erickson, G.F. 1980 Direct

inhibitory effect of gonadotropin-releasing hormone upon follicle-stimulating hormone induction of luteinizing hormone receptor and aromatase activity in rat granulosa cells. Endocrinology 106:1697-1705

Hsueh, A.J.W. and Jones, P.B.C. 1981 Extrapituitary actions of gonadotropin-releasing hormone. Endocr Rev 2:186-209

Hsueh, A.J.W. and Schaeffer, J.M. 1985 Gonadotropin-releasing hormone as a paracrine hormone and neurotransmitter in extrapituitary sites. J Steroid Biochem 23:757-764

Huckle, W. and Conn, P.M. 1987 The relationship between gonadotropin-releasing hormone-stimulated luteinizing hormone release and inositol phosphate production: studies with calcium antagonists and protein kinase C activators. Endocrinology 120:160-69

Hulting, A-L., Lindgren, J.A., Hokfelt, T., Eneroth, P., Werner, S., Patrono, C. and Samuelsson, B. 1985 Leukotriene C4 as a mediator of luteinizing hormone release from rat anterior pituitary cells. Proc Natl Acad Sci USA 82:3834-3838

Husain, A., Bumpus, F.M., De Silva, P. and Speth, R.C. 1987 Localization of angiotensin II receptors in ovarian follicles and the identification of angiotensin II in rat ovaries. Proc Natl Acad Sci USA 84:2489-2493

Ingraham, D.L. 1959 The effect of oestrogen on the atresia of ovarian follicles. J Endocrinol 19:123-125

Irvine, R.F. 1982 How is the level of free arachidonic acid controlled in mammalian cells. Biochem J 204:3-16

Irvine, R.F., Brown, K.D. and Berridge, M.J. 1984 Specificity of inositol trisphosphate-induced calcium release from permeablized Swiss-mouse 3T3 cells. Biochem J 222:269-272

Irvine, R.F., Letcher, A.J., Lands, D.J. and Downes, P. 1984 Inositol trisphosphates in carbachol-stimulated rat parotid glands. Biochem J 223:237-243

Jacobs, B.R. and Smith, R.G. 1980 Evidence for a receptor-like protein for progesterone in bovine ovarian cytosol. Endocrinology 106:1276-1282

components involved in adenylate cyclase inhibition by hormones. Adv Cyclic Nucleotide Protein Phosphorylat Res 17:135-143

Jones, P.B.C. and Hsueh, A.J.W. 1980 Direct inhibitory effect of gonadotropin-releasing hormone upon luteal luteinizing hormone receptor and steroidogenesis in hypophysectomized rats.

components involved in adenylate cyclase inhibition by hormones. Adv Cyclic Nucleotide Protein Phosphorylat Res 17:135-143

Jones, P.B.C. and Hsueh, A.J.W. 1980 Direct inhibitory effect of gonadotropin-releasing hormone upon luteal luteinizing hormone receptor and steroidogenesis in hypophysectomized rats. Endocrinology 107:1930-1936

Jones, P.B.C. and Hsueh, A.J.W. 1981a Direct stimulation of ovarian progesterone-metabolizing enzyme by gonadotropin-releasing hormone in cultured granulosa cells. J Biol Chem 256:1248-1254

Jones, P.B.C. and Hsueh, A.J.W. 1981b Direct effects of gonadotropin-releasing hormone and its antagonist upon ovarian functions stimulated by FSH, prolactin, and LH. Biol Reprod 24:747-759

Jones, P.B.C. and Hsueh, A.J.W. 1982a Pregnenolone biosynthesis by cultured granulosa cells: modulation by follicle-stimulating hormone and gonadotropin-releasing hormone. Endocrinology 111:713-721

Jones, P.B.C. and Hsueh, A. J. W. 1982b Regulation of ovarian 3 -hydroxysteroid dehydrogenase activity by gonadotropin-releasing hormone and Follicle-stimulating Hormone in cultured rat granulosa cells Endocrinology 1982:1663-1671

Judd, A.M., Jarvis, W.D. and MacLeod, R.M. 1987 Attenuation of pituitary polyphos[hoinositide metabolitsm by protein kinase C activation. Molecul Cellul Endocrinol 54:107-114

Kaibuchi,KY., Takai, Y, Ogawa, Y, Kimura, S, Nishizuka, Y, Nakamura, T, Tomomura, A and Ichihara, A. 1981 Inhibitory action of adenosine-3',5'-monophosphate on phosphatidylinositol tunrover: difference in tissue response. Biochem Biophys Res Commun 104:105-112

Kasson, B.G., Conn, P.M. and Hsueh, A.J.W. 1985 Inhibition of granulosa cell differentiation by dioctanoylglycerol-a novel activator of protein kinase C Molecul cellul Endocrinol 42:29-37

Kasson, B.G., Median, R., Davoren, J.B. and Huseh, A.J.W. 1985 Identification of subpopulations of rat granulosa cells: sedimentation properties and hormone responsiveness. Endocrinology 117:1027-1034

Katz, Y. and Armstrong, D.T. 1976 Inhibition of ovarian estradiol secretion by luteinizing hormone in prepubertal, pregnant mare serum-treated rats. Endocrinology 99:1442-1447

Kautsky, M.P. and Hagerman, D.D. 1980 Kinetic properties of steroid 19-hydroxylase and estrogen synthetase from porcine ovary microsomes. J Steroid Biochem 13:1283-1290

Kawai, Y. and Clark, M.R. 1985 Phorbol ester regulation of rat granulosa cell prostaglandin and progesterone accumulation Endocrinology 116:2320-2326

Kawai, Y. and Clark, M.R. 1986 Mechanism of action of gonadotropin releasiing hormone on rat granulosa cells. Endocr Res 12:195-209

Kennedy, T.G. 1979 Prostaglandin and increased endometrial vascular permeability resulting from the application of an artificial stimulus to the uterus of the rat sensitized for the decidual cell reaction. Biol Reprod 20:560-566

Kiesel, L., Przylipiak, A., Rabe, T. and Runnebaum, B. 1986 The role of novel arachidonic acid metabolites in GnRH action on gonadotrophin release in vitro. Human Reprod 1:349-352

Kiesel, L., Przylipiak, A., Rabe, T., Przylipiak, M. and Runnebaum, B. 1987 Arachidonic acid and its lipoxygenase metabolites stimulate prolactin release in superfused pituitary cells. Human Reprod 2:281-285

Kiesel, L., Przylipiak, A., Rabe, T. and Runnebaum, B. 1987 Leukotrienes stimulate gonadotropin release in vitro. Gynecol Endocrinol 1:23-35

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. 1980 Actions of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. J Biol Chem 255:2273-2276

Knecht, M. and Catt, K. 1981 Gonadotropin-releasing hormone: regulation of adenosine 3',5'-monophosphate in ovarian granulosa cells Science 214:1346-1348

Knecht, M., Katz, M. and Catt, K.J. 1981 GnRH inhibits cyclic nucleotide accumulation in cultured rat granulosa cells. J Biol Chem 256:34-36

Knecht, M., Amsterdam, A. and Catt, K. 1982 Inhibition of granulosa cell differentiation by gonadotropin-releasing hormone. Endocrinology 110:865-872

Knecht, M., Ranta, T., Naor, Z. and Catt, K.J. 1983 Direct effects of GnRH on the ovary. In: Factors regulating Ovarian Function, edited by G.S. Greenwald and P.F. Terranova. pp. 225-243

Knecht, M., Ranta, T., Feng, P., Shinohara, O. and Catt, K.J.

1985 Gonadotropin-releasing hormone as a modulator of ovarian functions. J Steroid Biochem 23:771-778

Kojima, I., Kojima, K. and Rasmussen, H. 1985 Characteristics of angiotensin II-, K^+-, and ACTH-induced calcium influxin adrenal glomerulosa cells. Evidence that angiotensin II, K+-, and ACTH may open a common calcium channel. J Biol Chem 260:9177-9184

Kolena, J. and Channing C.P. 1972 Stimulatory effects of LH, FSH and prostaglandin upon 3',5'-AMP levels in porcine granulosa cells. Endocrinology 90:1543-1550

Kolesnick, R.N., Musacchio, I. and Thaw, C. 1984 Arachidonic acid mobilizes calcium and stimulates prolactin secretion from GH3 cells. Am J Physiol 246:E458-462

Koos, R. and Clark, M.R. 1982 Production of 6-keto-prostaglandin F-1-alpha by rat granulosa cells in vitro. Endocrinology 11:1513-1518

Koos, R.D. and LeMaire, W.J. 1983 Factors that may regulate the growth anf regression of blood vessels in the ovary. Sem Reprod Endocrinol 1:295-307

Koos, R.D. and LeMaire, W.J. 1985 The effects of gonadotropin-releasing hormone agonist on ovulation and steroidogenesis during perfusion of rabbit and rat ovaries in vitro. Endocrinology 116:628-632

Kraft, A.S., Anderson, W.B., Cooper, H.L. and Sando, J.J. 1982 Decreases in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. J Biol Chem 257:3193-3196

Lacroix, E., Eechaute, W and Leusen, I. 1974 The biosynthesis of oestrogen by cow follicles. Steroids 23:337-356

Lahav, M., West, L.A. and Davis, J.S. 1988 Effect of prostaglandin F2alpha and a gonadotropin-releasing hormone agonist on inosotol phospholipid metabolism in isolated rat corpora lutea of various ages Endocrinology 123:1044-1052

Lapetina, E.G., Billah, M.M. and Cuatrecasa, P. 1981 The phosphadidylinositol cycle and the regulation of arachidonic acid production. Nature 292:367-369

Lapetina, E.G., Billah, M.M. and Cuatrecasas, P. 1981 The initial actions of thrombin on platelets. Conversion of phosphatidylinositol to phosphatidic acid preceding the production of arachidonic acid. J Biol Chem 265:5037-5040

Lawrence, Jr. I.E. and Burden, H.W. 1980 The origin of the

extrinsic adrenergic innervation to the rat ovary. Anat Rec 196:51-59

Leaver, H.A. and Boyd, G.S. 1981 Control of steroidogenesis in rat corpus luteum: the rate of access of cholesterol to the active site of the cholesterol side-chian cleavage enzyme. J Endocrinol 91:123-133

Lehninger, A.L. 1970 Mitochondrial and calcium ion transport. Biochem J 119:129-138

LeMaire, W.J. and Marsh, J.M. 1975 Interrelationships between prostaglandins, cyclic AMP and steroids in ovulation. J Reprod Fertil (Suppl) 22:53-74

Leung, P.C.K., Goff, A.K., Kennedy, T.G. and Armstrong, D.T. 1978 An intraovarian inhibitory action of estrogen and androgen production in vivo. Biol Reprod 19:641-647

Leung, P.C.K. and Armstrong, D.T. 1979 Estrogen treatment of immature rats inhibits ovarian androgen production in vitro. Endocrinology 104:1411-1417

Leung, P.C.K. 1985 Mechanism of gonadotropin-releasing hormone and prostaglandin action on luteal cells. Can J Physiol Pharmacol 63:249-256

Leung, P.C.K., Minegishi, T., Ma, F., Zhou, F.Z. and Ho Yuen, B. 1986 Induction of polyphosphoinositide breakdown in rat corpus luteum by prostaglandin F2a. Endocrinology 119:12-18

Leung, P.C.K., Wang, J. and Baimbridge, G.K. 1989 Role of calcium in the action of LHRH on ovarian progesterone production. VII Ovarian Workshop (in press)

Leung, P.K.C., Raymond, V. and Labrie, F. 1983 Stimulation of phosphatidic acid and phosphatidylinositol labeling in luteal cells by luteinizing hormone-releasing hormone. Endocrinology 112:1138-1140

Leung, P.K.C., Minegishi, T. and Wang, J. 1988 Inhibition of FSH and cyclic adenosine-3',5'-monophosphate induced progesterone production by calcium and protein kinase C in the rat ovary. Am J Obstet Gynecol 158:350-356

Li, C.H., Yamashiro, R.D. and Chung, D. 1987 Gonadotropin-releasing peptide from human follicular fluid: isolation, characterization, and chemical synthesis. Proc Natl Acad Sci USA 84:959-962

Liebeman, S., Greenfield, N.J. and Wolfson, A. 1984 A heuristic proposal for understanding steroidogenesis process. Endocr Rev 5:128-148

Limor, R., Ayalon, D., Capponi, A.M, Childs, G.V. and Naor, Z. 1987 Cytosolic free calcium levels in cultured pituitary cells separated by centrifigal elutriation: effect of gonadotropin-releasing hormone. Endocrinology 120:497-503

Lin, T. 1985 Mechanism of action of gonadotropin-releasing hormone-stimulated Leydig cell steroidogenesis. III. The role of arachidonic acid and calcium/phospholipid dependent protein kinase. Life Sci 36:1255-1264

Lindner, H.R., Tsafriri, A., Lieberman, M.E., Zor, U., Koch, Y., Bauminger, S. and Barne, A. 1974 Gonadotropin action on cultured granulosa cells: Induction of maturationdivision of the mammalian oocyte and differentiation of the luteal cell. Rec Prog Hormone Res 30:79-138

Lindner, H.R., Zor, U., Kohen, F., Bauminger, S., Armsterdam, A., Lahar, M. and Salomon, Y. 1980 Significance of prostaglandins in the regulation of cyclic events in the ovary and uterus. Adv Prostaglandin Thromboxane Leukotriene Res 8:1371-1390

Lino, J., Baranao, S. and Hammond, J.M. 1985 Multihormone regulation of steroidogenesis in cultured porcine granulosa cells: studies in serum-free medium. Endocrinology 116:2143-2151

Lynch, C.J., Charest, R., Bocckino, S.B., Exton, J.H. and Blackmore, P.F. 1985 Inhibition of hepatic al-adrenergic effects and binding by phorbol myristate acetate. J Biol Chem 260:2844-2851

Ma, F. and Leung, P.C.K. 1985 Luteinizing hormone-releasing hormone enhances polyphosphoinositide breakdown in rat granulosa cells. Biochem Biophys Res Commun 130:1201-1208

Madej, E. 1980 Effect of exogenous hormones on the acticity of delta5,3beta-hydroxysteroid dehydrogenase in cultured granulosa cells from proestrous and preovulatory rat ovarian follicles. Acta Histochem 67:253-260

Magnusson, C. and LeMaire, W.J. 1981 A gonadotropin-releasing hormone agonist stimulates oxygen consumption and maturation of follicle-enclosed rat oocytes in vitro. Acta Physiol Scand 111:377-379

Magoffin, D.A., Reynolds, D.S. and Erickson, G.F. 1981 Direct inhibitory effect of GnRH on androgen secretion by ovarian interstitial cells. Endocrinology 109:661-663

Magoffin, D.A. and Erickson, G.F. 1982 Mechanism by which GnRH inhibits androgen synthesis directly in ovarian interstitial cells. Mol Cell Endocrinol 27:191-198

Makris, A. and Ryan, K.J. 1975 Progesterone, androstenedione, testosterone, estrone, and estradiol synthesis in hamster ovarian follicle cells. Endocrinology 96:694-701

Makris, A. and Ryan, K.J. 1980 The source of follicular androgen in the hamster follicle. Steroids 35:53-64

Marchetti, B. and Cioni, M. 1988 Opposite changes of pituitary and ovarian receptors for LHRH in ageing rats: further evidence for a direct neural control of ovarian LHRH receptor activity. Neuroendocrinology 48:242-251

Marian, J. and Conn, P.M. 1983 Subcellular localization of the receptor for gonadotropin-releasing hormone in pituitary and ovarian tissue. Endocrinology 112:104-112

Marsh, J.M. and Savard, K. 1966 The stimulation of progesterone synthesis in bovine corpora lutea by adenosine-3',5'-monophosphate. Steroids 8:133-148

Marsh, J.M., Yang, N.S.T. and LeMaire, W.J. 1974 Stimulation of prostaglandin synthesis by LH in graafian follicles in vitro. Endocrinology (suppl) 56:A109

Mason, N.R., Schaffer, R.J. and Toomey, R.E. 1973 Stimulation of cyclic AMP accumulation in rat ovaries in vitro. Endocrinology 93:34-41

Massicotte, J., Veilleux, R., Lavoie, M. and Labrie, F. 1980 An LHRH agonist inhibits FSH-induced cyclic AMP accumulation and steroidogenesis in porcine granulosa cells in culture. Biochem Biophys Res Commun 94:1362-1366

McNatty, K.P., Sawers, R.S. and McNeilly, A.S. 1974 A possible role for prolactin in control of steroid secretion by the human Graafian follicle. Nature 250:635-655

McPhail, L.C., Clayton, C.C. and Snyderman, R. 1984 A potential second messenger role for unsaturated fatty acids: actions of Ca2+-dependent protein kinase. Science 224:622-625

Milvae, R.A., Murphy, B.D. and Hansel, W. 1984 Prolongation of the bovine estrous cycle with a gonadotropin-releasing hormone analog. Biol Reprod 31:664-670

Milvae, R.A., Alila, H.W. and Hansel, W. 1986 Involvement of lipoxygenase products of arachidonic acid metabolism in bovine luteal function. Biol Reprod 35:1210-1215

Minegishi, T. and Leung, P.C.K. 1985 Luteinizing hormone-releasing hormone stimulates arachidonic acid release in rat granulosa cells. Endocrinology 117:2001-2007

Minegishi, T., Wang, J. and Leung, P.C.K. 1987 Luteinizing hormone-releasing hormone (LHRH)-induced arachidonic acid release in rat granulosa cells: role of calcium and protein kinase C. FEBS Lett 214:139-142

Mohsin, S. and Pennefather, J.N. 1979 The sympathetic innervation of the mammalian ovary. A review of pharmacological and histological studies. Clin Exp Pharm Physiol 6:335-354

Monaco, L., Adamo, S. and Conti, M. 1988 Follicle-stimulating hormone modulation of phosphoinositide turnover in the immature rat sertoli cell in culture. Endocrinology 123:2032-2039

Moon, Y.S. and Duleba, A.J. 1982 Comparative studies of androgen metabolism in thecr and granulosa cells of human follicles in vitro. Steroids 39:419-430

Morris, H.R., Taylor, G.W., Jones, C.M., Piper, P.H., Samhoun, M.N. and Tippins, J.R. 1982 Slow reacting substances (leukotrienes): enzymes involved in their biosynthesis. Proc Natl Acad Sci USA 79:4838-4842

Moyle, W.R., Kuczek, T. and Bailey, C.A. 1985 Potential of a quantal Response as a mechanism for oscillatory behavior: Implications for our concepts of hormonal control mechanisms. Biol Reprod 32:43-69

Murakami, k. and Routtenberg, A. 1985 Direct action of purified protein kinase C by unsaturated fatty acids (oleate and arachidonate) in the absence of phospholipids and Ca2+. FEBS Lett 192:189-193

Murdoch, W.J., Dailey, R.A. and Inskeep, E.K. 1981 Preovulatory changes in prostaglandins E2 and F2 in ovine follicles. J Animal Sci 53:192-205

Naess, O. 1981 Characterization of cytoplasmic progesterone receptors in rat granulosa cells. Evidence for nuclear translocation. Acta Endocrinol (Capenh) 98:288-294

Naor, Z. and Catt, K.J. 1981 Mechanism of action of gonadotropin-releasing hormone: involvement of phospholipid turnover in luteinizing hormonr release. J Biol Chem 256:2226-2229

Naor, Z. and Yavin, E. 1982 Gonadotropin-releasing hormone stimulates phospholipid labeling in cultured granulosa cells. Endocrinology 111:1615-1619

Naor, Z., Azrad, A., Limor, R., Zakut, H. and Lotan, M. 1986 Gonadotropin-releasing hormone activates a rapid Ca2+-independent phosphodiester hydrolysis of polyphosphoinositides in pituitary gonadotrophs. J Biol Chem 261:12506-12512

Naor, Z., Vanderhoek, J.Y., Lindner, H.R. and Catt, K.H. 1983 Arachidonic acid products as possible mediators of the action of gonadotropin-releasing hormone. In: Advances in Prostaglandin, Thromboxane, and Leukotriene Research 12:259-263

Navickis, R.J., Jones, P.B.C. and Hsueh, A.J.W. 1982 Modulation of prolactin receptors in cultured rat granulosa cells by FSH, LH and GnRH. Mol Cell Endocrinol 27:77-88

Nimrod, A. 1977 Studies on the synergistic effect of androgen on the stimulation of progestin secretion by FSH in cultured rat granulosa cells: A search for the mechanism of action. Mol Cell Endocrinol 8:201-211

Nimrod, A., Rosenfield, R.L. and Otto, P. 1980 Relationship of androgen action to androgen metabolism in isolated rat granulosa cells. J Steroid Biochem 13:1015-1019

Nishizuka, T. 1984 The role of protein kinase C in cell surface transduction and tumor promotion Nature 308:693-698

Nishizuka, Y., Takai, Y., Kishimoto, A., Kikkawa, U. and Kaibuichi, K. 1984 Phospholipid turnover in hormone action. Recent Prog Horm Res 40:301-341

Nishizuka, Y. 1986 Studies and perspectives of protein kinase C. Science 233:305-312

Noland, T.A. and Dimino, M.J. 1986 Characterization and distribution of protein kinase C in ovarian tissue. Biol Reprod 35:863-872

O'Doherty, J., Youmans, S.J., Armstrong, W. McD. and Stark, R.J. 1980 Calcium regulation during stimulus secretion coupling: continuous measurement of intracellular calcium activites. Science 209:510-513

Orellana, S.A., Solski, P.A. and Brown, L.H. 1985 Phorbol ester inhibits phosphoinositide hydrolysis and calcium mobilization in cultured astrocytoma cells. J Biol Chem 260:5236-5239

Oron, Y., Dascal, N., Nadler, E. and Lupu, M. 1985 Inositol 1,4,5-triphosphate mimics muscarinic response in Xenopus oocytes. Nature 313:141-143

Ossowski, L., Biegel, O. and Reich, E. 1979 Mammary plasminogen activator: Correlation with involution, hormonal modulation and comparison between normal and neoplastic tissues. Cell 16:929-940

Pace-Asciak, C.R. and Smith, W.L. 1986 Enzymes in the biosynthesis and catabolism of the eicosanoids: prostaglandins, thromboxanes, leukotrienes and hydroxy fatty acids, Enzymes

16:543-603

Patwardhan, V.V. and Lanthier, A. 1981 Prostaglandin E and prostaglandin F in human ovarian follicles: Endogenous contents and in vitro formation by theca and granulosa cells. Acta Endocrinol 97:543-550

Pelletier, G., Seguin, C., Dube, D. anf St.-Arnaud, R. 1982 Distribution of LHRH receptors in the rat ovary. Biol Reprod (Suppl) 26:151A

Pellicer, A., Palumbo, A., DeCherney, A. H. and Naftolin, F. 1988 Blockage of ovulation by an angiotensin antagonist Science 240:1660-1661

Peters, H., Byskov, A.G., Himelstein-Braw, R. and Faber, M. 1975 Follicular growth: the basic event in the mouse and human ovary. J Reprod Fertil 45:559-566

Philibert, D., Ojasoo, T. and Raynaud, J-P. 1977 Properties of the cytoplasmic progestin-binding protein in the rabbit uterus. Endocrinology 101:1850-1861

Phillips, H.S., Hostetter, G., Kerdelhue, B. and Kozlowski, G.P. 1980 Immunocytochemical localization of LHRH in central olfactory pathways of hamster. Brain Res 193:574-579

Philpott, J.E. and Peron, F.G. 1971 A microassay procedure for 3beta-hydroxysteroid dehydrogenase based on substrate depletion. Endocrinology 88:1082-1085

Popkin, R.M., Bramly, T.A., Currie, A.J., Shaw, R.W., Baird, D.T. and Fraser, H.M. 1983 Specific binding of luteinizing hormone-releasing hormone to human luteal tissue. Biochem Biophys Res Commun 114:750-756

Prentki, M., Biden, T.J., Janjic, D., Irvine, R.F., Berridge, M.J. and Wollheim, C.B. 1984 Rapid mobilization of Ca^{2+} from rat insulinoma microsomes by inositol-1,4,5-trisphosphate. Nature 309:562-564

Prentki, M., Corkey, B.E. and Matschinsky, F.M. 1985 Inositol 1,4,5,-trisphosphate and the endoplasmic reticulum Ca^{2+} cycle of a rat insulinoma cell line. J Biol Chem 260:9185-9190

Putney, J.W., Jr., McKinney, J.S., Aub, D.L. and Leslie, B.A. 1984 Phorbol ester-induced protein secretion in rat parotid gland. Relationship to the role of inositol lipid breakdown and protein kinase C activation in stimulus-secretion coupling. Mol Pharmacol 26:261-266

Putney, J.W., Jr. 1986 A model for receptor-regulated calcium entry. Cell Calcium 7:1-12

Ranta, T., Baukal, A., Knecht, M., Korhonen, M. and Catt, K. J. 1983 Inhibition actions of a gonadotropin-releasing hormone agonist on ovarian follicle-stimulating hormone receptors and adenylatte cyclase in vivo. Endocrinology 112:956-963

Ranta, T., Knecht, M., Darbon, J-M., Baukal, A.J. and Catt, K.J. 1983 Calcium dependence of the inhibitory effect of gonadotropin-releasing hormone on luteinizing hormone-induced cyclic AMP production in rat granulosa cells. Endocrinology 113:427-429

Rasmussen, H. 1983 Pathways of Amplitude and Sensitivity Modulation in the Calcium Messenger System. In: Calcium and Cell Function, edited by W.Y. Cheung. pp 1-63

Rasmussen, H. and Barrett, P.Q. 1984 Calcium messenger system: an integrated view. Physiol Rev 64:938-984

Raymond, V., Leung, P.C.K., Veilleux, R., Lefevre, G. and Labrie, F. 1984 LHRH rapidly stimulates phosphatidylinositol metabolism in enriched gonadotrophs. Mol Cell Endocrinol 36:157-164

Reich, R., Abisogun, A.O., Miskin, R. and Tsafriri, A. 1985 The mechanism(s) of LH and GnRH induced steroidogenesis and plasminogen activator in preovulatory follicles. J Steroid Biochem (Suppl) 23:5

Reich, R., Kohen, F., Naor, Z. and Tsafriri, A. 1983 Possible involvement of lipoxygenase products of arachidonic acid pathway in ovulation. Prostaglandin 26:1011-1020

Reich, R., Kohen, F., Slager, R. and Tsafriri, A. 1985 Ovarian lipoxygenase activity and its regulation by gonadotropin in the rat. Prostaglandins 30:581-590

Reynolds, E.E. and Dubyak, G.R. 1985 Activation of calcium mobilization and calcium influx by alpha 1-adrenergic receptors in a smooth mulscle cell line. Biochem Biophys Res Commun 130:627-632

Richards, J.S. 1975 Estradiol receptor content in rat granulosa cells during follicular development: modification by estradiol and gonadotropins. Endocrinology 97:1174-1184

Richards, J.S., Ireland, J.J., Rao, M.C. and Bernath, G.A. 1976 Ovarian follicular development in the rat: hormone receptor regulation by estradial, follicle stimulating hormone and luteinizing hormone. Endocrinology 99:1562-1570

Richards, J.S. and Williams, J.J. 1976 Luteal cell receptor content for prolactin (PRL) and luteinizing hormone (LH): Regulation by LH and PRL. Endocrinology 99:1571-1581

Richards, J.S. and Bogovich, K. 1982 Effect of human chorionic gonadotropin and progesterone on follicular development in the immature rat. Endocrinology 111:1429-1438

Richards, J.S. and Hedin, L. 1988 Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. Ann Rev Physiol 50:441-463

Rink, T.J., Smith, S.W. and Tsien, R.Y. 1982 Intracellular free calcium in platelete shape change and aggregation. J Physiol 324:53-54

Rippel, R.H. and Johnson, E.S. 1976 Inhibition of hCG-induced ovarian and uterine weight augmentation in the immature rat by analogs of GnRH. Proc Soc Exp Biol Med 152:432-436

Rivier, C., Rivier, J. and Vale, W. 1986 Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. Science 234:205-208

Rodbell, M. 1980 The role of hormone receptors and GTP-regulatory proteins in mambrane transduction. Nature 284:17-22

Rolland, R. and Hammond, J.M. 1975 Demonstration of a specific receptor for prolactin in porcine granulosa cells. Endocr Res Commun 2:281-298

Rolland, R., Hammond, J.M. and Schellekens, L.A. 1976 Prolactin and ovary function. In: The Endocrine function of the human ovary, edited by James, V.H. et al., 1976. pp.305-321

Roth, J., LeRoith, D., Schiloach, J. and Rabinowitz, C. 1983 Intercellular communication: an attempt at a unifying hypothesis. Clin Res 31:354-363

Rothchild, I. 1981 The regulation of the mammalian corpus luteum. Recent Prog Horm Res 37:183-298

Rush, J.S. and Waechter, C.J. 1987 Inhibitors of protein kinase C block activation of B lymphocytes by bacterial lipopolysaccharide. Biochem Biophys Res Commun 145:1315-1320

Salari, H., Braquet, P. and Borgeat, P. 1984 Comparative effects of indomethacin, acetylenic acid, 15-HETE, nordihydroguaiaretic acid and BW 755C on the metabolism of arachidonic acid in human leukocytes and platelets. Prostaglandins leukotriences Med 13:53-59

Samuelsson, B. 1983 Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 220:568-575

Samuels, L.T., Helmreich, M.L., Lasater, M.B. and Reich, H.

1951 An enzyme in endocrine tissues which Oxidizes delta5, 3beta hydroxysteroids to a,b nusaturated ketones. Science 113:490-491

Schaeffer, J.M. and Hsueh, A.J.W. 1982 Identification of gamma-aminobutyric acid and its binding sites in the ovary. Life Sci 30:1599-1604

Schreiber, J.R. and Erickson, G.F. 1979 Progesterone receptor in the rat ovary: further characterization and localization in the granulosa cell. Steroids 34:459-469

Shears, S.B. and Kirk, C.J. 1984 Determination of mitochondrial calcium content in hepatocytes by a rapid cellular fractionation technique. Vasopressin stimulates mitochondrial Ca++ uptake. Biochem J 220:417-421

Sheldrick, E.L. and Flint, A.P.F. 1984 Ovarian oxytocin. In: Gonadal Proteins and Peptides and Their Biological Significance, edited by M.R. Sairam and L.E. Atkinson. pp. 257-272

Shinohara, O., Knecht, M., and Catt, K.J. 1985 Inhibition of gonadotropin-induced granulosa cell differentiation by activation of protein kinase C Proc Natl Acad Sci USA 82:8518-8522

Shinohara, O., Kencht, M., Feng, P. and Catt, K.J. 1986 Activation of protein kinase C potentiates cyclic AMP production and stimulates steroidogenesis in differentiated ovarian granulosa cells. J Steroid Biochem 24:161-168

Short, R.V. 1962 Steroids in the follicular fluid and the corpus luteum of the mare: A 'two-cell type" theory of ovarian steroid synthesis. J Endocrinol 24:59-63

Smith C. and Perks A.M. 1983 The kinin system and ovulation: changes in plasma kininogens, and in kinin-forming enzymes in the ovaries and blood of rats with 4-day estrous cycles. Can J Physiol Pharmacol 61:736-742

Smith, J.B., Smith, L. and Higgins, B.L. 1985 Temperature and nucleotide dependence of calcium release by myo-inositol 1,4,5-trisphosphate in cultured vascular smooth muscle cells. J Biol Chem 260:14413-14416

Smith, M.S. 1980 Role of prolactin in mammalian reproduction. Int Rev Physiol 22:249-276

Snyder, G.D. and Bleasdale, J.E. 1982 Effect of LHRH on incorporation of [32P]orthophosphate into phosphatidylinositol by dispersed anterior pituitary cells. Mol Cell Endocrinol 28:55-63

Somlyo, A.P., Bend, M. and Somlyo, A.V. 1985 Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly in vivo. Nature 314:622-625

Strauss III, J.F., Schuler, L.A., Rosenblum, M.F. and Tanaka, T. 1981 Cholesterol metabolism by ovarian tissue. Adv Lipid Res 18:99-157

Streb, H., Bayerdorfer, E., Haase, W., Irvine, R.F. and Schulz, I. 1984 Effect of inositol 1,4,5-trisphosphate on isolated subcellular fractions of rat pancreas. J Membr Biol 81:241-253

Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. 1983 Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306:67-69

Strickland, S. and Beers, W.H. 1976 Studies on the role of plasminogen activator in ovulation. In vitro response of granulosa cells to gonadotropin, cyclic nucleotides and prostaglandins. J Biol Chem 251:5694-5702

Sulimovici, S. and Boyd, G.S. 1968 The cholesterol side-chain cleavage enzyme in immature rat ovary. Eur J Biochem 3:332-345

Sulimovici, S. and Boyd, G.S. 1969 The delta5, 3beta-hydroxysteroid dehydrogenase of rat ovarian tissue. Eur J Biochem 7:549-558

Sullivan, M.H. and Cooke, B.A. 1986 The role of Ca^{2+} in steroidogenesis in Leydig cells. Stimulation of intracellular free Ca^{2+} by lutropin (LH), luliberin (LHRH) agonist and cAMP. Biochem J 236:45-51

Takai, Y., Kikkawa, U., Kaibuchi, K. and Nishizuka, Y. 1984 Membrane phospholipid metabolism and signal transduction for protein phosphorylation. In: Advances in Cyclic Nuceotide and Protein Phosphorylation Research, edited by Greengard, P. and Robinson, G.A. pp. 119-157

Takats, A. and Hertelendy, F. 1982 Adenylate cyclase activity of avian granulosa: effect of gonadotropin-releasing hormone. Gen Comp Endocrinol 48:515-524

Taylor, M.V., Metcalfe, J.C., Hesketh, T.R., Smith, G.A. and Moore, J.P. 1984 Mitogens increase phosphorylation of phosphoinositides in thymocytes. Nature 312:462-465

Terris, S., Hofmann, C. and Steiner, D.F. 1979 Mode of uptake and degradation of ^{125}I-labeled insulin by isolated hepatocytes and H4 hepatoma cells. J Cell Biol 71:159-171

Tilly, J.L. and Johnson, A.L. 1988 Effects of a phorbol ester,

a calcium ionophore, and 3',5'-adenosine monophosphate production on hen granulosa cell plasminogen activator activity Endocrinology 123:1433-1440

Toaff, M.E., Schleyer, H. and Strauss, III., J.F. 1982 Metabolism of 25-hydroxycholesterol by rat luteal mitochondria and dispersed cells. Endocrinology 111:1785-1790

Toaff, M.E., Strauss, J.F. III. and Hammond, J.M. 1983 Regulation of cytochrome P450 SCC in immature porcine granulosa cells by FSH and estradiol. Endocrinology 112:1156-1158

Touqui, L., Rothhut, B., Shaw, A.M., Fradin, A., Vargaftig, B.B. and Russo-Marie, F. 1986 Platelet activation-a role for a 40K anti-phospholipase A_2 protein indistinguishable from lipocortin. Nature 321:177-180

Triebwasser, W.F., Clark, M.R., LeMaire, W.J. and Marsh, J.M. 1978 Localization and in vitro synthesis of prostaglandins in components of rabbit preovulatory graafian follicles. Prostaglandins 16:621-632

Tsafriri, A., Lindner, H.R., Zor, U. and Lamprecht, S.A. 1972 Physiological role of prostaglandins in the induction of ovulation. Prostaglandins 2:1-10

Tsafriri, A., Lindner, H.R., Zor, U. and Lamprecht, S.A. 1972 In vitro induction of meiotic division in follicle-enclosed rat oocytes by LH, cyclic AMP and prostaglandin E2. J Reprod Fertil 31:39-50

Tsafriri, A. and Braw, R. 1984 Experimental approaches to atresia in mammals. Oxford Rev Reprod Biol 6:226-265

Tsafriri, A., Reich, R. and Abisogun, A.O. 1986 Ovarian regulation of oocyte maturation. In: Gynecological Endocrinology, edited by Genazzani, A.R., Volpe, A. and Facchinetti, F. pp 109-115

Tsang, B.K., Moon, Y.S., Simpson, C.W. and Armstrong, D.T. 1979 Androgen biosynthesis in human ovarian follicles: cellular source, gonadotropic control, and andenosine 3',5'-monophosphate mediation. J Clin Endocrinol Metab 48:153-158

Tsang, B.K., Armstrong, D.T. and Whitfield, J.F. 1980 Steroid biosynthesis by isolated human ovarian follicular cells in vitro. J Clin Endocrinol Metab 51:1407-1411

Tureck, R.W., Mastroianni, L, Jr., Blasco, L. and Strauss, J.F. 1982 Inhibition of human granulosa cell progesterone secretion by a GnRH agonist. J Clin Endocrinol Metab 54:1078-1083

Uchida, K., Kadowaki, M., Miyake, T. and Wakabayashi, K. 1972

Effect of exogenous progesterone on the ovarian progestin secretion and plasma LH and prolactin levels in cyclic rats. Endocrinol Jpn 19:323-333

Veldhuis, J., Kolp, L.A., Toaff, M.E., Strauss, III, J.F. and Demmers, L.M. 1983 Mechanism subserving the tropic actions of insulin on ovarian cells. In vitro studies using swine granulosa cells. J Clin Invest 72:1046-1057

Veldhuis, J. D. and Demers, L. M. 1986 An inhibitory role of the protein kinase C pathway in ovarian steroidogenesis Biochem. J. 239:505-511

Veldhuis, J.D. and Demers, L.M. 1987 Activation of protein kinase C is coupled to prostaglandin F_{2a} synthesis in the ovary: studeis with cultured swine granulosa cells. Mol Cell Endocrinol 49:249-254

Vilgrain, I., Defaye, G. and Chambaz, E.M. 1984 Adrenocortical cytochrome P-450 responsible for cholesterol side chian cleavage (P-450 scc) is phosphorylated by the calcium-activated, phospholipid-sensitive protein kinase (protein kinase C). Biochem Biophys Res Commun 125:554-561

Volpi, M., Molski, T.E.P., Naccache, P.H., Feinstein, M.B. and Sha'afi, R.I. 1985 Phobol 12-myristate 13-acetate potentiates the action of the calcium ionophore in stimulating arachidonic acid release and production of phosphatidic acid in rabbit neutrophils. Biochem Biophys Res Commun 128:594-600

Wang, C. 1983 Luteinizing hormone releasing hormone stimulates plasminogen activator production by rat granulosa cells. Endocrinology 112:1130-1132

Wang, C. and Hsueh, A.J.W. 1979 Induction of functional prolactin receptors by follicle-stimulating hormone in rat granulosa cells in vitro. J Biol Chem 254:11330-11336

Wang, C., Hsueh, A.J.W. and Erickson, G.F. 1981 LH stimulation of estrogen secretion in cultured granulosa cells. Mol Cell Endocrinol 24:17-28

Wang, J., Baimbridge, K.G. and Leung, P.C.K. 1989 Perturbation of intracellular calcium ion concentration in single rat granulosa cells by angiotensin II. Endocrinology (in press).

Wang, J. and Leung, P.C.K. 1987 Role of arachidonic acid in luteinizing hormone-releasing hormone actions: stimulations of progesterone production in rat granulosa cells. Endocrinology 122:906-911

Wang, J. and Leung, P.C.K. 1987 Role of protein kinase C in luteinizing hormone-releasing hormone (LHRH)-stimulated

progesterone production in rat granulosa cells. Biochem Biophys Res Commun 146:939-944

Watson, S.P., McConnell, R.T. and Lapetina, E.G. 1984 The rapid formation of inositol phosphate in humane platelets by thrombin is inhibitied by prostacyclin. J Biol Chem 259:13199-13203

Welsh, Jr. T.H., Jones, P.B.C., Ruiz, de Galarreta, C.M., Fanjul, L.F. and Hsueh, A.J.W. 1982 Androgen regulation of progestin biosynthetic enzymes in FSH-treated rat granulosa cells in vitro. Steroids 40:691-700

Welsh, Jr., T.H., Jones, P.B.C. and Hsueh, A.J.W. 1984 Phorbol ester inhibition of ovarian and testicular steroidogenesis in vitro. Cancer Research 44:885-892

Wolfe, M., LeVine, III H., May, S. Jr., Cuatrecacasa, P. and Sahyoun, N. 1985 A model for intracellular translocation of protein kinase C involving synergism between calcium and phorbol esters. Nature 317:546-49

Yamamoto, S., Ishii, M., Nakadate, T., Nakaki, T. and Kato, R. 1983 Modulation of insulin secretion by lipoxygenase productions of arachidonic acid. J Biol Chem 258:12149-12152

Ying, S.Y. and Guillemin, R. 1979 (D-Trp6-Pro9-NEt)-luteinizing hormone-releasing factor inhibits follicular development in hypophysectomized rats. Nature 280:593-595

Zoller, L.C. and Weisz, J. 1978 Identification of cytochrome P450 and its distribution in the menbrana granulosa of the preovulatory follicle using quantitative cytochemistry. Endocrinology 103:310-313

Zoller, L.C. and Weisz, J. 1979 A quantitative cytochemical study of glucose-6-phosphate dehydrogenase and 3beta-hydroxysteroid dehydrogenase activity in membrana granulosa of the ovulable type of follicle of the rat. Histochemistry 62:125-135

Zor, U., Strulovici, B., Braw, R., Lindner, H.R. and Tsafriri, A. 1983 Follicle-stimulating hormone-induced prostaglandin E formation in isolatedrat ovarian theca. J Endocrinology 97:43-49

PUBLICATIONS

Leung PCK, Minegishi T and Wang J 1988 Inhibition of follicle-stimulating hormone- and adenosine-3',5'-cyclic monophosphate-induced progesterone production by calcium and protein kinase C in the rat ovary. Am J Obstet Gynecol 158:350-356

Leung PCK and Wang J 1989 Inositol lipids and LHRH action in the ovary. J Reprod Fertil (suppl) 37:287-293

Leung PCK and Wang J 1989 Role of calcium in the action of LHRH on ovarian progesterone production. In: VII ovarian workshop: Paracrine Communication in the ovary (in press).

Leung PCK and Wang J 1989 The role of inositol lipid metabolism in the ovary. Biol Reprod (in press).

Leung PCK, Wang J, and Baimbridge KG 1989 Mechanism of action of luteinizing hormone-releasing hormone in rat ovarian cells Can J Physiol Pharmacol (in press).

Minegishi T, Wang J and Leung PCK 1987 Luteinizing hormone-releasing hormone (LHRH)-induced arachidonic acid release in rat granulosa cells: Role of calcium and protein kinase C. FEBS Lett 214:139-142

Wang J, Baimbridge KG, and Leung PCK 1989 Perturbation of interacellular calcium ion concentrations in single rat granulosa cells by angiotensin II. Endocrinology 124:1094-1096

Wang J, Baimbridge KG, and Leung PCK 1989 Changes in cytosolic free calcium ion concentrations in individual rat granulosa cells: effect of luteinizing hormone-releasing hormone. Endocrinology 124:1912-1917.

Wang J, Ho Yuen B, and Leung PCK 1989 Stimulation of progesterone and prostaglandin E_2 production by lipoxygenase metabolites of arachidonic acid. FEBS Lett 244:154-158.

Wang J, Lee V and Leung PCK 1989 Differential role of protein kinase C in the action of LHRH on hormone production in rat ovarian cells. Am J Obstet Gynecol (in press).

Wang J and Leung PCK 1987 Role of protein kinase C in luteinizing hormone-releasing hormone (LHRH)-stimulated progesterone production in rat granulosa cells. Biochem. Biophys. Res. Comm. 146:939-944

Wang J and Leung PCK 1988 Role of arachidonic acid in luteinizing hormone-releasing hormone action: stimulation of progesterone production in rat granulosa cells. Endocrinology 122:906-911

Wang J and Leung PCK 1989 Arachidonic acid as a stimulatory mediator of LHRH action in the rat ovary. Endocrinology 124: 1973-1979

Wang J and Leung PCK 1989 Synergistic stimulation of prostaglandin E_2 production by calcium ionophore and protein kinase C activator in rat granulosa cells. Biol Reprod (in press).