MORPHOLOGY, STEROIDOGENESIS AND ENZYME ACTIVITY

IN LARGE AND SMALL PORCINE LUTEAL CELLS

A Thesis

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Wei Yuan

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WEI YUAN

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The current studies investigated the effects of FSH, LH, oxytocin, $PGF_{2\alpha}$ and PKC activity on steroidogenesis in the isolated LC and SC on days 10 and 15 of the estrous cycle. As well, the morphology of the LC and SC was examined on days 10 and 15 of the estrous cycle, and days 30 and 60 of pregnancy using TEM and SEM.

The LC and SC were enzymatically dissociated from porcine CL at day 10 or 15 of the estrous cycle and incubated in Ham's F12 nutrient media containing 10% heat deactivated calf serum and the designated treatment compound. TEM and SEM used isolated cells from the cycle as well as luteal tissue slices from pregnancy. The current studies demonstrated for the first time that there were two kinds of LC, one containing small and nonfingerprint SER; the other having large, tubular and fingerprint SER, which occupied approximately 40% of the area in the cell. The former were referred to as alpha LC and the latter as beta LC. The number of beta LC was greater in the CL from pregnancy than during the estrous cycle. This indicates that alpha LC can differentiate into beta LC. As well, the LC contained numerous microvilli which consisted of numerous micronetworks on the membrane. The SC contained primarily mitochondria, a small amount of SER and microvilli in both cyclic and pregnant CL. The number of mitochondria in the SC increased from pregnant CL.

FSH inhibited P4 production by the LC on day 15 of the

estrous cycle. This unique effect of FSH may be of benefit to follicle development by inhibiting P4 production from CL. Oxytocin and $PGF_{2\alpha}$ in short-term (2 to 3 h) incubation tended to increase P4 production in the LC on day 10, but decreased P4 production, specifically by the SC, on day 15 of the estrous cycle. TEM indicated that the LC still contained mitochondria, SER and lipid droplets on day 15 of the estrous cycle, but these had almost disappeared in the SC. This and the strong inhibition of P4 production by oxytocin and $PGF_{2\alpha}$ in day 15 SC suggested that luteolysis in the late luteal phase may be initiated in the SC.

The incubation of the LC and SC with PMA resulted in a translocation of PKC activity to the plasma membrane in the LC and left cytosolic PKC activity deficient. The SC was unique in that the detectable cytosolic PKC activity was greater than total PKC activity.

PMA increased P4 production in the LC and SC on day 10 but decreased P4 production on day 15 in short-term (2 h) incubation. This indicates that activation of PKC had a stimulatory effect on day 10 and an inhibitory effect on day 15. Testosterone and 17ß-estradiol production were not affected. H-7 blocked PMA effect and 4α-phorbol ester was without effect. $PGF_{2\alpha}$ and oxytocin also stimulated P4 production in the mid luteal phase and inhibited P4 production in the late luteal phase in a similar manner to PMA. This suggested that $PGF_{2\alpha}$, and possibly oxytocin, can affect the

porcine luteal P4 production through the PKC system. Long-term (12-24 h) incubation with PMA resulted in cytosolic PKC deficiency, lowered P4 production but no alteration in testosterone or 17ß-estradiol synthesis.

Progesterone production is specifically sensitive to PKC activity because 4α-phorbol ester did not influence P4 production. Inhibitor of PKC (H-7) blocked the effects of PKC on P4 production. Activation of PKC did not influence testosterone and 17ß-estradiol production.

DEDICATION

This thesis is dedicated to my wife, X.Y. Huang, in appreciation for her constant support, encouragement and patience during my Ph.D. program, and to my sons, Z.Y. Yuan and R.H. Yuan.

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FOREWORD

The preparation of this thesis followed a manuscript format. Manuscript I has been accepted for publication by the "Journal of Animal Science". Manuscript II has been submitted to "Biology of Reproduction and Manuscript III will be submitted to "Endocrinology". The authors of Manuscript I are W. Yuan, M.L. Connor and M.M. Buhr. The authors of Manuscripts II and III are W. Yuan and M.L. Connor.

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ABBREVIATIONS

SYMBOL

NAME

AA   Arachidonic Acid

BSA   Bovine Serum Albumin

CL   Corpus Luteum

cpm   Counts Per Minute

CV   Coefficient of Variation (%)

°C   Degree Centigrade or Celsius

cPKC   Conventional Protein Kinase C

dbcAMP   Dibutyryladenosine 3':5'-cyclic

              Monophosphate

DG   1,2-Dioleoyl-sn-Glycerol

DMSO   Dimethyl Sulphoxide

EDTA   Ethylenediamino Tetraacetic Acid

EGTA   [(Ethylenenebis-Oxyethylenenitrilo)]

                 Tetraacetic Acid

FSH   Follicle-Stimulating Hormone

G   Gram(s)

GLM   General Linear Models

h   Hour(s)

hCG   Human Chorionic Gonadotropin

H-7   1-[5-Isoquinolinylsulfonyl]

              -3-Methylpiperazine Dihydrochloride

H-8   (N-[2-(Methylamino)ethyl] 5-

              Isoquinolinesulfonamide Dihydrochloride)

LC	Large Luteal Cell(s)
LDL	Low Density Lipoprotein
LGC	Luteinized Granulosa Cells
LH	Luteinizing Hormone
LTC	Luteinized Theca Cells
min	Minute(s)
ml	Millilitre(s)
MSI	Manuscript I
MSII	Manuscript II
MSIII	Manuscript III
ng	Nanogram(s) $(gX10^{-9})$
nPKC	Novel Protein Kinase C
P4	Progesterone
$P450_{scc}$	C27 Side Chain Cleavage Enzyme
PBS	Phosphate Buffer Saline
pg	Picogram(s) $(gX10^{-12})$
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI2	Prostacyclin I 2
PI	Phosphatidylinositol
PIP	Phosphatidylinositol 4-Phosphate
PIP2	Phosphatidylinositol 4,5-Biphosphate
IP3	1,4,5-Inositol Triphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PRL	Prolactin

pLH	Porcine Luteinizing Hormone
PS	$1\text{-}\alpha$-Phosphatidyl-$_L$-Serine
RIA	Radioimmunoassay
SC	Small Luteal Cell(s)
sem	Standard Error of Mean
SEM	Scanning Electron Microscopy
SER	Smooth Endoplasmic Reticulum
TEM	Transmission Electron Microscopy
TNF	Tumor Necrosis Factor
3ß-HSD	3ß-Hydroxysteroid Dehydrogenase

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INTRODUCTION

The development of improved and economical methods of controlling reproduction in pigs and other farm animals depends heavily on an increased understanding of the physiological mechanisms regulating ovarian function. The swine CL is unique because it is not susceptible to the luteolytic influence of $PGF_{2\alpha}$ <u>in vivo</u> in the mid-luteal phase (Connor et al., 1976; Britt, 1987) and shows a minimal luteotropic response to LH <u>in vitro</u> (Hunter, 1981; Buhr, 1987; Agu, 1991). However, these unique features and their mechanisms are not well understood. Furthermore, the role of the putative luteotropic (FSH and LH) and luteolytic hormones ($PGF_{2\alpha}$) in the regulation of porcine CL function have not been well defined.

Oxytocin may participate in luteolysis <u>in vivo</u> (Hansel et al., 1987; Fuchs, 1988). However, the effect of oxytocin on P4 production by luteal cells <u>in vitro</u> is controversial (Tan et al., 1982; Mukhopadhyay et al., 1984; Richardson and Masson, 1985; Przala et al., 1986). Most of these studies indicated that oxytocin had little effect (Fuchs, 1988). The effect of oxytocin on porcine LC and SC has not been characterized. $PGF_{2\alpha}$ has demonstrated both luteotropic and luteolytic properties <u>in vitro</u> (Alila et al., 1988 a,b; Wiesak, 1991). The effects noted depend on the species and stage of the cycle. However, any direct effect of $PGF_{2\alpha}$ on porcine luteal

steriodogenesis _in vitro_ is not clear.

It is generally considered that LH plays its role in steriodogenic cells through cAMP, Ca^{++} and cAMP-dependent PKA. However, the function of cAMP as the second messenger has recently been questioned because LH stimulates P4 production only in bovine and ovine SC, but not in LC (Hansel et al., 1987; Niswender and Nett, 1988). Neither porcine LC or SC are sensitive to LH on day 10 _in vitro_ (Hunter, 1981; Buhr, 1987; Agu, 1991).

The Ca^{++}-polyphosphoinositol-dependent PKC second messenger systems have been implicated as having an important role in steroidogenesis in ovarian tissue of various species (Hansel et al., 1987). Activation of PKC stimulated P4 production in bovine luteal cells (Brunsuig et al., 1986; Alila et al., 1988) but inhibited P4 production in ovine (Hoyer et al., 1989; Wiltbank et al., 1989) and rat (Baum and Rosberg, 1987) luteal cells. However any differences of PKC activity related to cell type in porcine luteal tissues and PKC effect on steroidogenesis have not been reported.

Studies have indicated that LC from porcine CL produced much more P4 than SC on day 10 of the estrous cycle (Buhr, 1987), but any morphological differences between LC and SC related to P4 production have not been demonstrated.

The present investigations were designed to study :

1) the effects of LH, FSH, oxytocin and $PGF_{2\alpha}$ on P4 production in LC and SC on days 10 and 15 during the estrous

cycle.

2) the morphological characteristics of porcine LC and SC during the estrous cycle and early to mid-pregnancy using SEM and TEM.

3) total and cytosolic PKC activity in LC and SC on days 10 and 15 of the estrous cycle using natural and pharmacologic activators.

4) P4, testosterone and 17ß-estradiol production when PKC was activated using a pharmacologic activator, phorbol-12-myristate 13-acetate (PMA) in LC and SC on days 10 and 15 during the estrous cycle.

Corpora lutea on days 10 and 15 of the cycle were specifically selected for the current studies because porcine CL are relatively unresponsive to luteolytic and luteotropic hormones on day 10, but on day 15 are more susceptible to luteolysins such as $PGF_{2\alpha}$.

REVIEW OF THE LITERATURE

In this chapter, morphology and physiology of the corpus luteum in farm animals will be primarily reviewed. Most advanced achievements and areas of disagreement in the studies of the corpus luteum will be discussed.

General Morphology of Ovary

As in other farm animals, ovaries develop and mature when pigs enter puberty. The ovary of cattle and sheep is almond-shaped, whereas the ovary of horse is bean-shaped and has a definite ovulation fossa. The porcine ovary looks like a cluster of grapes in which follicles and corpora lutea (CL) obscure the underlying ovarian tissue. The ovary consists of two parts: the medulla and cortex and is surrounded by the superficial epithelium. The medulla is composed of connective tissue, nervous and vascular systems. The cortex contains follicles and / or CL at various stages of development or regression (Hafez, 1987).

Physiology of the Corpus Luteum

Early History of Origin of the Corpus Luteum

In the late 1800s and early 1900s, there were two hypotheses concerning the follicle cell type responsible for the formation of the CL (reviewed Hansel et al., 1987). One suggested that CL were derived exclusively from granulosa cells of the follicle while the cells from the theca interna degenerated shortly after ovulation. The other one held exactly the opposite view; stating that both the theca interna and granulosa layers of the ovarian follicle were involved in the formation of CL. The first report of an ingrowth of thecal cells into the CL of the sow was made by Corner in 1919 and is considered as a classic paper on the development of porcine CL (Hansel et al., 1987). It is now commonly accepted that both theca and granulosa cells contribute to development of the CL but their relationship to specific cell types in the CL is not clear.

Formation and Development of the Corpus Luteum

Generally, there are about 50 small follicles in the two ovaries during the early follicular phase of the estrous cycle and luteal phase. Ten to twenty follicles develop and approach the tertiary or Graafian follicle stage (preovulatory follicles). The Graafian follicle contains a large single antrum filled with liquor folliculi, which occupies the thickness of the cortex and protrudes from the surface of the ovary. The cavity is lined by many layers of granulosa cells. Cells of the ovarian stroma which surround the follicular cells form the theca folliculi. The theca folliculi contain two layers of cells: the fibrous theca externa and the vascular theca interna.

Immediately after ovulation, the follicle walls collapse in folds, and the cavity is filled with clotted blood, and the structure is called the corpus hemorrhagicum. The granulosa and theca cells proliferate rapidly and develop into luteal cells which form the CL. Within six to eight days, the CL, consisting of a solid mass of luteal cells and highly vascularized connective tissue, reaches its maximal weight. The CL exists until day 16 of the estrous cycle. After day 16, the CL begins to degenerate. It decreases in size and becomes a white or pale brown scar of connective tissue known as the corpus albicans (Hafez, 1987).

Function of the Corpus Luteum

The CL produces several peptide and steroid hormones such as oxytocin, relaxin, P4, 17ß-estradiol, testosterone and prostaglandins (PGs), but the primary function of the CL is to synthesize and secrete P4. Progesterone plays an important role in the reproductive system and prepares this system for the support of pregnancy. The following will describe briefly the effects of P4 in the reproductive systems during the estrous cycle and pregnancy.

During the luteal phase of the estrous cycle, high levels of P4 inhibits uterine contractions and stimulates growth and development of the endometrium, especially the endometrial glands to secrete endometrial fluid for blastocyst implantation. Progesterone is required for the continuous maintenance of pregnancy in most mammals, at least during the first one-third of gestation. Progesterone has antiestrogen effects including myometrial hyperpolarization, and decreased sensitivity to oxytocin and decreased estrogen receptors. Progesterone is necessary for the growth of mammary glands, especially for lobular-alveolar development. So the CL plays its important role in the reproductive system through its production of progesterone.

Characteristics of Large and

Small Luteal Cells

Origin of Large and Small Luteal Cells

There are three main cell types in the CL: 1) small (< 10 μM) non-steroidogenic cells which consist mainly of vascular cells (endothelial cells, erythrocyte and leucocytes) and connective tissue cells such as fibrocytes; 2) small steroidogenic cells (10-20 μm) and 3) large steroidogenic cells (> 25 μm) (Hansel et al., 1987). Since LC and SC have at least two potential origins, thecal and granulosa cells, it is very interesting to understand the relationship between LC and SC. Evidence for the follicular origin of LC and SC is contradictory. Several histologic studies suggest that luteal cells are derived from both cell types of the follicle, the granulosa giving rise to LC and the theca interna to SC (Hansel et al., 1987).

Until recently, histochemical and histological studies were the best source of information on the follicular origin of the different cell types of the CL. However, these methods have inherent limitations since the rapid cellular rearrangement that occurs after ovulation makes it difficult to accurately distinguish the origin of the cells in the developing CL. Recently, monoclonal antibodies have been successfully used in dissecting complex cellular development

in several tissues. Alila and Hansel (1984) used specific monoclonal antibodies to surface antigens of preovulatory theca and granulosa cells to trace the origin of the cells in ovine CL. They found that the percent binding of granulosa antibodies to LC was influenced by the stage of the reproductive cycle. The majority of LC (77%) were bound by granulosa antibodies (GrAB) between days 4 and 6 of the estrous cycle. There was a significant decline in the percent of large cells bound as the age of the CL increased : 47.5% and 30.2% for day 10-12, respectively. About 14% of the SC bound to the GrAB between 4 and 6 of the estrous cycle. The theca antibodies did bind to a majority of SC throughout the estrous cycle. The theca antibody also bound to LC and the proportion of these cells increased significantly from 10% between days 4 and 6 to 46% by days 10 to 12 of the estrous cycle. Alila and Hansel (1984) proposed a model for the source of LC and SC and their development, which stated that all the SC were of thecal origin, and until the sixth day of the estrous cycle, nearly all the LC were of granulosa origin. After this time, LC of theca origin (derived from the SC) appeared. The granulosa-derived LC disappeared during early pregnancy, while the cells of the thecal origin persisted throughout pregnancy.

Recently the LC were subdivided into proposed α- and β-LC. The β-LC (40-46 μm) contained numerous mitochondria and a more apparent smooth endoplasmic reticulum (SER) than the

smaller α-LC (32-38 μm). In addition, ß-LC were more sensitive to $PGF_{2\alpha}$ as evidenced by their depletion of small granules and swelling of SER and mitochondria (Fields et al., 1991).

Difference of Fine Structures between Large and Small Cells

Porcine LC have highly convoluted membranes and numerous tubular SER (Belt et al., 1970; Gemmell, et al., 1973). In the case of SER, the membranes more frequently formed fenestrated cisternae in parallel array which were sometimes concentric. During the estrous cycle, SER was usually present as a system of fine anastomotic tubules and were only rarely present as organized cisternae (Belt et al., 1970). In bovine CL, both LC and SC contain abundant SER and rough ER, Golgi and lipid droplets. The SC have peripherally located and deeply lobulated nuclei with a distinct nucleolus and dispersed chromatin. Mitochondria, which are more abundant in the LC, surround the nucleus and are almost absent from the cell periphery. Their membrane surfaces are highly convoluted and contain extensive microvillus projections. The main distinguishing feature between LC and SC is the presence of electron dense granules of various sizes in the cytoplasm of some LC; the cytoplasm of all small cells is devoid of these granules which have been suggested to contain some of the progesterone (Hansel et al., 1987). During the pregnancy, LC contain the granules enclosed by a common membrane and the intramitochondrial dense bodies, which are absent from LC of

the estrous cycle. The presence of glycogen bodies is a unique feature of SC in the bovine CL (Hansel et al., 1987).

The fine structure of the CL in the ewe (Paavola and Christensen, 1981; O'Shea, 1987) is similar to that in sows and cows. The unique feature in ovine luteal cells is that they contain small, densely staining granules appearing within the luteal cells on day 2 of the estrous cycle, and then increasing in number as the cycle progresses. Maximum secretion of granules takes place at days 10 and 11, and this is followed by a progressive decline in secretory activity from day 12 to day 15 of the cycle. Recently, Fields et al. (1991) found that $PGF_{2\alpha}$ caused a release of oxytocin into the circulation and a decline in secretory granules on day 7 that were not replenished by day 12. Irrespective of treatment, all secretory granules contained oxytocin and neurophysin. The results suggested that in the early part of the cycle a single cluster of secretory granules containing oxytocin and neurophysin are formed and once secretory granules are depleted, the cell is incapable of generating a second population of secretory granules.

Location of Hormones in Luteal Cells

In addition to progesterone and other steroid hormones, the CL produces a variety of hormones. Oxytocin, relaxin, neurophysin, PGs and insulin like growth factor-I (IGF-1) have been found in the CL. Many lines of evidence now indicate that

oxytocin is synthesized, stored and secreted by LC but not by SC in ewe (Rodgers et al., 1983; Sawyer and Moeller, 1985). Immune-electron microscopy has provided definitive proof of the granular localization of oxytocin and neurophysin-1 in ovine LC (Theodosis et al., 1986). In bovine LC, the granules contain oxytocin and neurophysin. However, it is not known whether each granule has several hormones or whether each hormone is in a different granule (Hansel et al., 1987).

It has been demonstrated using the immunoperoxidase method with electron microscopy that relaxin is present in bovine LC from the middle third of pregnancy (Fields et al., 1980). In pigs, relaxin is found to be present in the densely staining granules of LC during the estrous cycle (All et al., 1986) and pregnancy (Fields and Fields, 1985). There is little information about relaxin storage in the ewe (O'Shea, 1987). Relaxin plays an important role in late pregnancy especially at delivery time. However, little is known about the function of relaxin during the estrous cycle.

Steroidogenic Capability in Large and Small Cells

The LC produces more P4 than the SC. In the bovine, the LC account for less than 10% of the steroidogenic cells but the LC produce much more P4 than the SC (Hansel et al., 1987; Hansel et al., 1991). The ovine LC produces 47 times more P4 than the SC (Wiltbank et al., 1991). In pigs, production of P4 by the LC always exceeds that of the SC (Buhr, 1987).

Effects of LH and FSH on Luteal Cells

Chemistry of FSH and LH

The anterior pituitary gland secretes FSH and LH (Reeves, 1987). LH and FSH are polypeptides and both of them are composed of chains of amino acids linked together by peptide bonds with chains of carbohydrates linked to the polypeptides. LH and FSH consist of α and ß subunits. The α subunit is identical within species for FSH and LH. The molecular weight of LH and FSH is approximately 32,000 daltons, with each subunit having a molecular weight of 16,000 daltons.

LH contains an α and ß subunit with a molecular weight of 32,000 daltons and half-life (t1/2) of 20 min. The α subunit of FSH has 92 amino acids with carbohydrate side chains at amino acid 52 and 78; the ß subunit has between 108 to 118 amino acids with 2 carbohydrate side chains at amino acid 7 and 24. Both of the subunits are important to FSH and LH biological activity and if one of them is removed, FSH and LH will lose their biological activity. In addition, the carbohydrate fraction affects FSH and LH biological activity since they can extend the circulatory half-life of FSH and LH. Ovine and bovine LH α subunit is identical. Porcine LH α subunit is about 95% similar and human LH α subunit is about 75% similar to ovine LH α. The ß subunit imparts both hormone and species specificity. FSH also has a common α subunit and

a hormone-species specific ß subunit. Recently, six different species of FSH were isolated from a single animal, thus it appears that each of these FSH molecules may have a different biological activity (Reeves, 1987). The t1/2 of FSH is approximately 2 to 2.5 h.

Effects of LH and FSH during the Folliclar Phase

FSH primarily stimulates follicular development and maturation, and causes estrogen production. Proliferation of granulosa cells and synthesis of LH receptors by granulosa cells prior to luteinization is regulated by the synergistic actions of FSH and 17ß-estradiol (Hadley, 1988). There are two centres which control LH secretion: the tonic and preovulatory centres in the hypothalamus. Tonic or basal levels of FSH and LH induce estrogen secretion from the large Graafian follicles. The preovulatory surge of LH is responsible for luteinization of the granulosa cells and rupture of the mature follicle wall and ovulation.

Function of FSH and LH during the Luteal Phase

Luteinizing hormone is generally considered an important luteotropin in numerous species. However, the LC and the SC in the CL respond to LH differently. In the cow and ewe, the SC are more responsive to added LH than the LC (Fitz et al., 1982; Rodgers et al., 1984; Hansel et al., 1987). This fact is

consistent with the much higher numbers of LH receptors on the SC than the LC in ewe CL (33260 per SC compared with 3074 per LC) (Fitz et al., 1982). The results above indicate that P4 secretory response to LH occurs largely in the SC in ovine and bovine.

Responses in porcine luteal cells are much less clear. Porcine luteal cells had a minimal response to LH in vitro (Hunter, 1981; Buhr, 1987; Agu, 1991). Other studies have reported that LH can increase P4 secretion in both LC and SC of pregnant sows (Lemon and Loir, 1977) or in perfused porcine luteal cells(Li et al., 1991). Gregorazczuk (1992) reported that PRL and FSH formed a luteotropic complex in the early luteal phase (0 to 3 days after ovulation). However, PRL and FSH as well as LH did not have any effect on P4 production in the mid luteal phase. In the late luteal phase, these hormones increased 17ß-estradiol production. Porcine granulosa cells from early follicles responded to FSH by increasing P4 production (Romanoff, 1966) but granulosa cells from large follicles did not respond to FSH (Hylka and diZerega, 1990).

Hansel and Seifart (1976) found that purified bovine LH and other LH-containing preparations overcame the inhibitory effects of concurrently injected oxytocin on luteal tissue weight, P4 content and concentration. Prolactin, FSH and growth hormone could not overcome the inhibitory effects of oxytocin. FSH alone was without effect on luteal tissue weight, P4 content and concentration when administered on day

7 of the bovine estrous cycle (Hansel and Seifart, 1976).

Progesterone secretory pulses occurred more frequently than LH pulses whereas every FSH pulse was followed by a P4 pulse during the early or midluteal phase in cattle (Walters et al., 1984). In addition, during midgestation in cattle, when LH pulses were absent, there was a close temporal relationship between pulses of FSH and P4. In vitro, FSH did not influence P4 production by dispersed bovine luteal cells from early in the cycle (Tsang et al., 1991) or rat (Adashi et al., 1981). FSH basal levels were higher than that of LH in pigs during the estrous cycle (Parvizi et al., 1976) but a role in luteal function has not been reported.

The Traditional Second Messenger System and Steroidogenesis

Synthesis of Progesterone in the Luteal Cells

In recent years, a lot of understanding has been achieved about pathways of steroidogenesis in the CL, but the mechanisms involved in the synthesis and secretion of P4 are still not clear. Cholesterol bound to low density lipoprotein (LDL) or high density lipoprotein (HDL) is the primary substrate for P4 synthesis. LDL or HDL combine with their receptors and transfer cholesterol to the cytosol. Within the cell, the LDL or HDL-receptor complex combine with lysosomes

and the cholesterol is released. The free cholesterol is either used for P4 synthesis or esterified. For P4 production, the cholesterol is transported to the mitochondria, where it is converted to pregnenolone by the C_{27} side-chain cleavage enzyme. Pregnenolone is then converted to P4 by 3ß-hydroxysteroid dehydrogenase/$\triangle$5,$\triangle$4-isomerase in the SER (Niswender and Nett, 1988).

Receptors for LH and FSH

The receptor for LH is considered to be a glycolipoprotein. The receptors for LH increase when the CL increase in size. In the ewe, the total number of receptors for LH increased 40-fold between days 2 and 14 of the cycle. There was a 6-fold increase in the number of receptors occupied by endogenous hormone and the weight of the CL and a 10-fold increase in serum P4 concentration during this same period. However, less than 0.5% of the total LH receptors were occupied by endogenous hormone. The total number of receptors for LH and the number occupied by endogenous LH decreased by 75% on day 16. During the early pregnancy, the numbers of total and occupied receptors were very similar to those observed during the midluteal phase of the cycle (Diekman et al., 1978). LH receptors on the SC were much more numerous than the LC in the ewe CL (Fitz et al., 1982). During the bovine estrous cycle, unoccupied luteal receptors increased during luteal development and decreased after luteal

regression. As well, the number of luteal receptors for LH and P4 production during the cycle were positively correlated in primates, women, rats, sheep, horses and cattle (Niswender and Nett, 1990).

In pigs, the concentration of unoccupied receptors on the CL doubled between days 6 and 10 of the cycle, and decreased between days 12 and 14, and increased 3-fold between days 20 and 30 of pregnancy. Concentration of receptors occupied by LH increased early in the estrous cycle, in parallel with the total receptor concentration. In pregnancy, the percentage occupancy dropped dramatically as the total receptor concentration increased between days 20 and 30 of pregnancy. Receptor affinity constants increased towards the end of the cycle and decreased between days 20 and 30 of pregnancy (Ziecik et al., 1980). These results would imply that LH has a physiologal function during the porcine estrous cycle as well as during pregnancy. However, the studies in vitro indicate that LH has little effect on P4 production by isolated porcine luteal cells (Buhr, 1987).

Receptors for FSH have been detected in the bovine (Manns et al., 1984) and hamster (Oxberry and Greenwald, 1982) CL but not in the porcine CL (Ziecik et al., 1988). In the bovine, the CL in the early and late cycle had more FSH receptors (Manns et al., 1984) but the FSH receptors were low in mid cycle. The biological significance has yet to defined.

The Traditional Second Messenger Theory

Because LH has been considered as a primary hormone in luteinization of the CL, most of the studies on the luteinization of the CL have involved the second messenger (cAMP) theory. The traditional second messenger theory is that LH stimulates P4 synthesis through a second messenger system (Smith, 1986), which involves LH (first messenger), a membrane-bound LH receptor, conversion of adenosine triphosphate (ATP) to adenosine -3', 5'-cyclic monophosphate (cAMP, second messenger) by adenylate cyclase, activation of a cAMP-dependent protein kinase A (PKA), and an intracellular response (steroidogenesis).

The model recently proposed by Niswender and Nett (1988) is that most cell types have receptors for multiple regulatory agents, some of which stimulate adenylate cyclase, while others are inhibitory. The stimulatory or inhibitory action of the hormone receptor complexes are mediated via two transducing or coupling proteins (G-protein). The protein complex involved in activating adenylate cyclase is called the Gs (Gilman, 1984), while the protein that inhibits adenylate cyclase is called Gi. Gs binds to the regulatory subunit of adenylate cyclase, resulting in activation of the catalytic subunit which converts ATP to cAMP. The cAMP binds to the receptor subunit of PKA, causing dissociation and activation of the catalytic subunit. The catalytic subunit of PKA alters the activity of various protein substrates via

phosphorylation, leading to activation of the these proteins and modification of the biologic response.

Increased PKA activity can influence the function of the luteal cells via several ways (Niswender and Nett, 1988). PKA may promote nuclear events, gene expression and protein synthesis, including a cholesterol-binding protein. The phosphorylation action of PKA results in activation of cholesterol esterase and $P450_{scc}$. $P450_{scc}$ has a key role in the steroidogenic enzyme pathway and is thought to be the rate-limiting factor in steroidogenesis. In addition, the phosphorylation of PKA can stimulate transport of cholesterol into the mitochondrion and may stimulate transport of pregnenolone out of the mitochondria and the uptake of LDL or HDL in some species. Finally, the LH is degraded in lysosomes. The receptors for LH and LDL are probably recycled to the plasma membranes.

Challenge to Traditional Second Messenger Theory

As described before, LC are only 10% of the steroidogenic cells and contain fewer receptors for LH (Fitz et al., 1982), but LC occupy most of the area of the CL (Parry et al., 1980) and produce the majority of P4. Treatment of LC with cholera toxin or forskolin (adenylate cyclase activator) in the ewe resulted in a dramatic increase in intracellular cAMP, but there was no concommitant increase in P4 production (Niswender and Nett, 1988). In bovine luteal tissues, there was no

correlation between the occupied LH receptor concentration and basal adenylate cyclase activity. Large ovine and bovine luteal cells were not sensitive to LH (Niswender and Nett, 1988; Hansel et al., 1991). In contrast, the SC in ruminants contain the majority of the LH receptors (Fitz et al., 1982) and are sensitive to LH (Hansel et al., 1991). This indicates that the traditional second messenger system is primarily involved in SC (Niswender and Nett, 1988).

More recently, the "constitutive" induction of $P450_{scc}$ was proposed to explain the unresponsiveness of LC to LH (Hansel et al., 1991; Hansel, personal communication, 1992). It has been reported for the rat that once $P450_{scc}$ mRNA is induced as a consequence of the LH/hCG surge it is constitutively maintained by luteinized cells in vivo (0 to 4 days) and in vitro (0 to 9 days) in the absence of gonadotropins, is susceptible to modulation by prolactin and is no longer regulated by cAMP. Direct addition of a PKA inhibitor (H-8) to the luteinized cell cultures did not alter $P450_{scc}$ mRNA in these cells, indicating mRNA for $P450_{scc}$ in rat ovarian luteal cells is mediated by cAMP-independent mechanisms (Oonk et al., 1989). In the bovine, upon establishment of luteinization (day 9), levels of $P450_{scc}$ were three times higher in granulosa than theca cells luteinized in vitro, and mRNA levels for $P450_{scc}$ were five times higher in luteinized granulosa cells compared to luteinized theca cells. On day 8 after luteinization $P450_{scc}$ mRNA was still present in the luteinized granulosa cells but

was undetectable in the theca derived cells. These results indicated that sustained P4 production by the luteinized granulosa cells resulted from continuous enzyme synthesis and mRNA transcription even in the absence of luteotropic agents (Aflalo et al., 1992). Failure of LH to stimulate luteal cell P4 production early in the cycle may occur because these cells are already producing at a maximal level (Hansel et al., 1991; Hansel, personal communication, 1992). Further research is needed to unravel the mechanism controlling progesterone production by the LC.

Effects of PGF$_{2\alpha}$ and Oxytocin on the Corpus Luteum

Synthesis and Chemistry of PGF$_{2\alpha}$

Prostaglandins, discovered in the early 1930s, are believed to be present in almost every tissue. PGF$_{2\alpha}$ is one member of the prostaglandins, which belong to a family of chemically related substances: the eicosanoids. Eicosanoids are produced by a cell's response to a variety of extrinsic stimuli. In addition, prostaglandins may be synthesized by cells in response to some hormones and can, therefore, be considered as intracellular second messengers in the actions of these hormones (Hadley, 1988).

Phospholipids are sources for prostaglandin synthesis. Prostaglandins are produced exclusively within the plasma

membrane of cells and derived mainly from arachidonic acid which is released from phospholipids by the action of phospholipases. The arachidonic acid may be utilized in one or more different pathways, depending on the tissue concerned and particular nature of the extrinsic stimulus. In one pathway (Hadley, 1988), arachidonic acid is converted to unstable endoperoxide intermediates by a group of enzymes termed the cycloxygenase system. These intermediates may be converted to one of a number of related products: PGE_2, $PGF_{2\alpha}$ and PGI_2. Arachidonic acid (AA) has been reported to play an important role in P4 production (Lafrance and Hansel, 1992). AA stimulated oxytocin production by bovine LC and P4 production by both LC and SC on day 8 of the estrous cycle. Inhibition of the lipoxygenase metabolic pathway by nordihydroguaianetic acid abolished the AA-induced release of both oxytocin and P4 production in the bovine CL (Lafrance and Hansel, 1992).

Effect of $PGF_{2\alpha}$ in Luteolysis

The luteolytic effect of $PGF_{2\alpha}$ has been demonstrated in cattle, sheep, goats, horses and guinea pigs (Niswender and Nett, 1988). Interestingly, $PGF_{2\alpha}$ did not cause luteolysis in pigs until day 12 after estrus (Connor et al., 1976; Britt, 1987). There are several proposed mechanisms for the luteolytic effect of $PGF_{2\alpha}$. First, $PGF_{2\alpha}$ can cause contraction of the ovarian artery during the period of luteal regression, which results in a rapid and dramatic decrease in luteal blood

flow (Niswender and Nett, 1988). Secondly, $PGF_{2\alpha}$ can decrease the number of receptors for LH and cause an uncoupling of the receptor for LH from adenylate cyclase (Fletcher and Niswender, 1982), thus preventing LH from playing any luteotropic role. Finally, $PGF_{2\alpha}$ can cause a cytotoxic effect (Silvia et al., 1984).

The exact mechanism of $PGF_{2\alpha}$ action remains unknown. Receptors for $PGF_{2\alpha}$ in dispersed porcine luteal cells increased gradually to day 12, reached a maximum on day 13 which was maintained through day 17 (Gadsby et al., 1990). However, the porcine CL does not respond to $PGF_{2\alpha}$ until day 12 of the estrous cycle (Connor et al., 1976). $PGF_{2\alpha}$ increased P4 production in bovine SC (Benhaim et al., 1987; Alila et al., 1988a,b) in vitro. In mixed, dispersed porcine luteal cells, $PGF_{2\alpha}$ enhanced P4 production in short-term incubation (Mattioli et al., 1985).

Leucocytes also play important roles in luteolysis, possibly in conjunction with $PGF_{2\alpha}$. During the luteal regression, leucocytes produce cytokines which include tumour necrosis factor-alpha (TNF) and interleukin-I. Leukocyte-cytokine interactions may lead to the release of an intraovarian luteolysin ($PGF_{2\alpha}$) (Wang and Riley, 1992). It has been reported that TNF stimulates $PGF_{2\alpha}$ production in bovine luteal cells (Townson and Pate, 1992). TNF is also reported to inhibit aromatase activity in porcine LC and SC (Pitzel et al., 1992).

Oxytocin and Its Effect

Chemistry of Oxytocin

Two short-chain peptides, oxytocin and vasopressin, are present within the pars nervosa of most mammalian species. As described in the previous chapter, these hormones are also present in the CL. The hormones consist of nine amino acids folded into a ring through a disulphide bridge at positions 1 and 6 of the molecule, leaving a terminal tripeptide side chain. The molecular difference between oxytocin and vasopressin is that vasopressin has a phenylalanine and an arginine at the 3 and 8 positions while oxytocin has isoleucine and leucine at the 3 and 8 positions, respectively. These two hormones are closely related structurally, but serve quite different physiological roles. Oxytocin, for example, controls milk release from the mammary gland and contraction of the uterus, whereas vasopressin is concerned with water balance.

Regulation of Ovarian Oxytocin Biosynthesis

The evidence demonstrates that ovulation seems to trigger the expression of the oxytocin neurophysin gene in bovine ovaries <u>in vivo</u> (Jungclas and Luck, 1986). There was no mRNA for oxytocin and neurophysin in the preovulatory follicle, but

one day after ovulation peak levels of the specific mRNA were found in the ruptured follicle. The mRNA levels decreased significantly 5 to 6 days after ovulation and were low throughout the second half of the estrous cycle. Bovine granulosa cells in culture spontaneously luteinized without the addition of LH and began to secrete both P4 and oxytocin (Jungclas and Luck, 1986), which indicated that mRNA for oxytocin had already been produced in the granulosa cells. However, the thecal cells do not themselves express the oxytocin gene, but significantly stimulate oxytocin secretion when co-cultured with granulosa cells.

Oxytocin Level and Its Change in Vivo

Basal P4 and oxytocin concentrations in sheep changed simultaneously during the estrous cycle (Sheldrick and Flint, 1981). These similarities between P4 and oxytocin were also found in cows (Walters et al., 1984). Luteal oxytocin concentrations are highest in sheep and cows, but primates and porcine luteal tissues contain lower values. Oxytocin in the CL varies cyclically in the sow (Pitzel et al., 1984). In the bovine CL, oxytocin is very low on the day of ovulation; after ovulation oxytocin increases slowly and after 3 days it increases rapidly. On day 11, a peak of oxytocin is found, followed by a rapid decline (Fuchs, 1988).

Effect of Oxytocin on The Corpus Luteum in Vivo and in Vitro

Oxytocin has been implicated in luteolysis in sheep, goats and cows *in vivo* (Fuchs, 1988). Oxytocin injections given repeatedly to sheep and goats shortened estrous cycle length and caused luteal regression. Conversely, immunization against oxytocin led to prolonged luteal cycles in sheep and goats (Cooke and Homeida, 1985). However, the results from different experiments on the effect of oxytocin on P4 production by luteal cells *in vitro* have been conflicting. Oxytocin at a low concentration stimulated bovine luteal cells to produce P4, but inhibited gonadotropin-induced P4 secretion at higher doses (Tan et al., 1982; Tan and Biggs, 1984).

In the bovine, low levels of oxytocin were most stimulative for P4 production in the early luteal phase (days 5-7) and decreased continuously between days 8 to 12 to days 15 to 18 *in vitro*. Pre-exposure with an oxytocin antagonist blocked the stimulatory effect of oxytocin but not the stimulatory effect of LH (Schams and Miyamoto, 1991). But oxytocin had little direct effect on P4 production by rat, human and bovine luteal cells (Mukhopadhyay et al., 1984; Richardson and Masson 1985; Hansel et al., 1987). In pigs, a low concentration of oxytocin inhibited P4 production but high doses of oxytocin had no suppressive effect on mixed luteal cells (Przala et al., 1986). Wuttke et al., (1990) using a microdialysis system which can be implanted into CL of sows found that $PGF_{2\alpha}$ and oxytocin stimulated P4 production in young

CL but inhibited P4 secretion in old CL.

Cooperation of Oxytocin and PGF$_{2\alpha}$ in Luteolysis

The uterus in goat, ewe, cow and sow regulates the life span of the CL. In sheep and goats, PGF$_{2\alpha}$ can release oxytocin from the ovary and oxytocin in turn can release PGF$_{2\alpha}$ from the uterus. These abilities vary during the cycle in parallel with uterine oxytocin receptor numbers. It has been suggested that a positive feedback loop operates during luteolysis between the ovary and uterus, with oxytocin secreted by the ovary and PGF$_{2\alpha}$ from the uterus as messengers (Flint and Sheldrick, 1986). The unique anatomical proximity of the uterine veins and ovarian arteries in these species permits the diffusion of PGF$_{2\alpha}$ from the uterine vein directly into the ovarian artery by a countercurrent diffusion mechanism allowing for PGF$_{2\alpha}$ to bypass the general circulation and avoid rapid destruction in the lungs.

In rats, oxytocin injections shortened the duration of pseudopregnancy from a mean of 13.5 days in the control group to 11.5 days in the oxytocin-treated group (Li and Chan, 1991). The shortening of pseudopregnancy of the oxytocin-treated group was associated with the premature functional regression of the CL. Both the ovarian and uterine PGF$_{2\alpha}$ levels increased with pseudopregnancy. This suggested that oxytocin is luteolytic in rats, and the luteolytic action of oxytocin is mediated via PG release from the uterus.

In the cow, $PGF_{2\alpha}$ seems not to be closely linked to oxytocin, since oxytocin has a luteolytic role only in the early luteal phase, whereas $PGF_{2\alpha}$ is effective in the mid- or late-luteal phase. Moreover, oxytocin in the bovine CL falls to low levels long before luteolysis is completed. Luteal oxytocin is low at the end of the estrous cycle when the largest and most frequent pulses of $PGF_{2\alpha}$ are observed in the ovarian venous plasma. In addition, intrauterine injection of a prostaglandin synthesis inhibitor, indomethacin, is without effect on oxytocin-induced shortening of luteal life-span (Milvae and Hansel, 1985).

Hansel and Dowd (1986) suggested that PGI-2, which is the major prostanoid produced by the cyclo-oxygenase pathway in the early CL, can play a luteotropic role, while products of the lipo-oxygenase pathway of arachidonic acid metabolism, in particular 5-hydroxyeicosatetraenoic acid (5-HETE), play a luteolytic role. Studies demonstrated that PGI-2 greatly increased P4 secretion when injected directly into the CL of cows, and PGI-2 added to luteal cells in vitro caused a dose-related increase in P4 production (Hansel et al., 1987). The biosynthesis of PGI-2 by luteal cells was the greatest in the early luteal phase and fell to low levels during the second half, reaching a nadir at estrus. Indomethacin given in the early luteal phase significantly shortened the life-span of the CL and decreased cycle length. A lipo-oxygenase pathway inhibitor (nordihydroguairetic acid) given in the late luteal

phase prolonged estrous cycle length and prevented the drop in luteal cell PGI-2 production. Oxytocin given in the early luteal phase suppressed PGI-2 biosynthesis by luteal cells very substantially, suggesting that the luteolytic effect of oxytocin may be regulated by inhibiting the synthesis of PGI-2 within the CL (Hansel and Dowd, 1986).

In summary, oxytocin is synthesized in the luteal cells of all species so far studied, and the oxytocin-neurophysin gene is expressed in granulosa cells and granulosa-derived luteal cells, but not in the theca cells. Oxytocin can inhibit P4 production _in vivo_. However, the results of the effect of oxytocin on P4 production _in vitro_ are conflicting; with most of the studies indicating that oxytocin has little direct effect on luteal P4 production. The concept that oxytocin from the ovary participates in the luteolytic process is gaining acceptance. In this context, ovarian oxytocin acts as a hormone inducing $PGF_{2\alpha}$ release from the uterus. $PGF_{2\alpha}$ in turn causes contraction of the ovarian artery, induces a rapid decrease in luteal blood flow and decreases numbers of receptors for LH. This may cause a cytotoxic effect leading to luteolysis.

DG, IP3 and PKC System and Steroidogenesis

Earlier in this review, the traditional second messenger system was discussed in the regulation of steroidogenesis in

the CL. Since the traditional second messenger system is primarily involved in LH, cAMP and PKA system in the SC (Niswender and Nett, 1988), it is therefore speculated that steroidogenesis in the CL, especially in the LC, is regulated by other second messenger systems. Another, less well demonstrated, second messenger system involves diacylglycerol (DG) and 1,4,5-inositol triphosphate (IP3). DG, once considered as an ordinary intermediate of glycerolipid synthesis and degradation, is now demonstrated to have a much greater role in eukaryotic cells. Importantly, DG is found to function as an intracellular second messenger, activating a phospholipid- and Ca^{++} dependent serine/threonine protein kinase , protein kinase C (PKC) (Berridge, 1987). PKC then could phosphorylate a variety of substrate proteins resulting in changes of cellular activities. IP3 can cause Ca^{++} mobilization which induces physiological responses. DG and IP3 have been implicated in numerous cellular processes, including regulation of cell growth, differentiation, secretion, activation of neutrophils, regulation of gene expression, cell surface receptors and cellular metabolism (Bell, 1986; Berridge, 1987). It has been demonstrated that LH, oxytocin, GnRH, $PGF_{2\alpha}$, insulin, thyrotropin-releasing hormone (TRH), vasopressin, neurotensin and angiotensin can promote increases in inositol lipid turnover in different tissues (Brown, 1987). This indicates that these hormones are related to DG, IP3 and PKC second messenger systems.

Synthesis and Chemistry of DG and IP3

A remarkable feature of the newly discovered second messenger system is that a phospholipid constituent of the membrane is itself used as the phosphorylated precursor for the production of the messenger molecules. Hormones or neurotransmitters interact with a receptor which activates the G-protein (Gilman, 1987). The G-protein then interacts with the membrane-bound phosphoinositide-specific phospholipase C enzyme, which involves the hydrolysis of the inositol lipid, phosphatidylinositol 4,5-bisphosphate (PIP2). PIP2 is a phospholipid bearing three phosphate groups and present primarily in the inner leaflet of the membrane. PIP2 is formed by the phosphorylation of phosphatidyl inositol (PI), which is also present predominantly in the inner leaflet of the plasma membrane and represents about 5% of the total membrane phospholipid (Figure 1, Brown, 1987). A phosphate is added to PI on the 4-position to give phosphatidylinositol 4-phosphate (PIP) by a PI kinase. PIP is further phosphorylated by a PIP kinase to yield PIP2. The phospholipase C hydrolyses PIP2 into DG and IP3. Both of them appear to function as second messengers: IP3 causing the release of Ca^{++} from bound intracellular stores, including the SER and mitochondria, and DG working as a potent activator of PKC (Berridge, 1987).
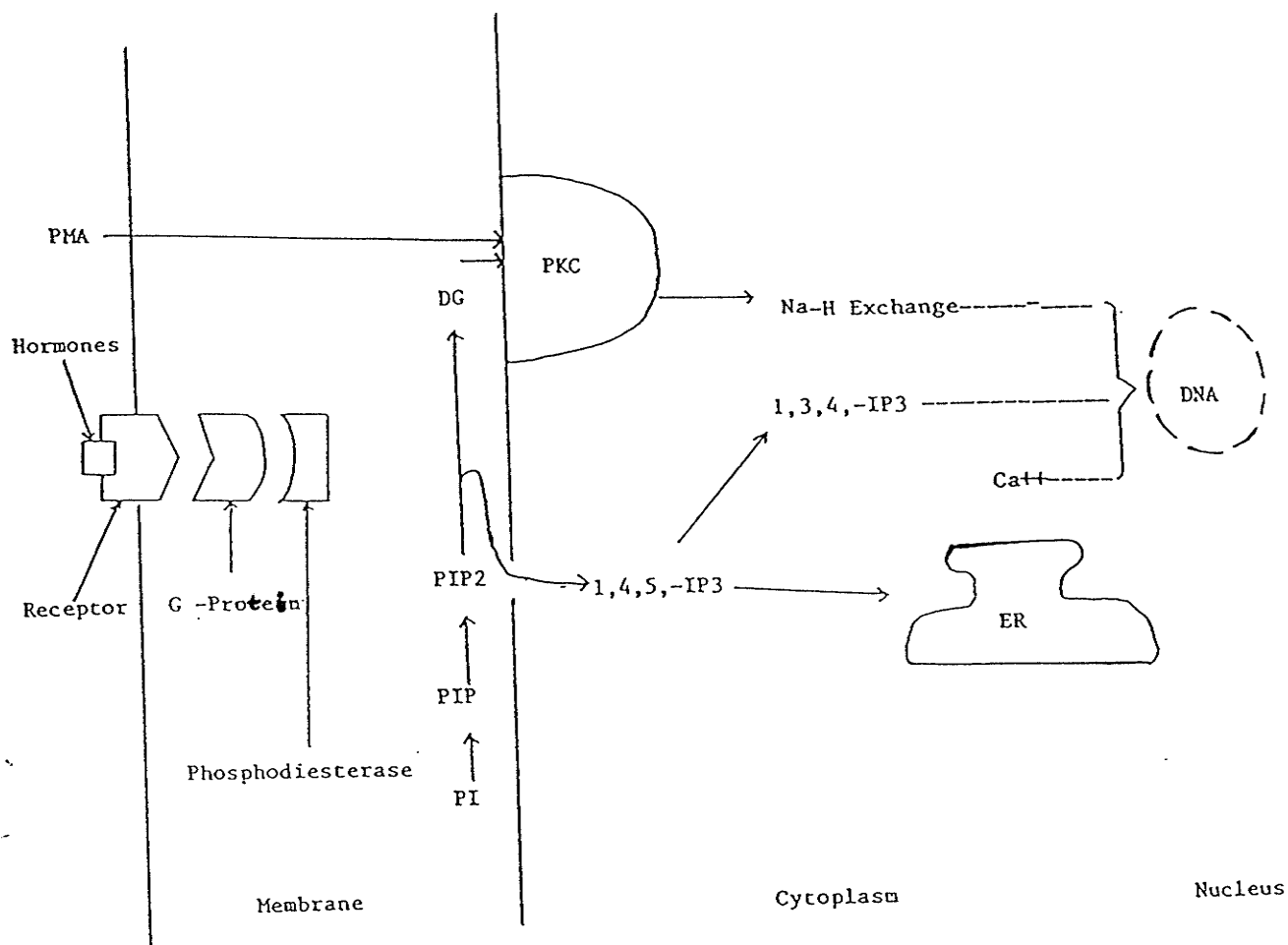
PI: Phosphatidylinositol

PIP: Phosphatidylinositol-4-phosphate

PIP2: Phosphatidylinositol-4,5-bisphosphate

IP3: Inositol-1,4,5-triphosphate

DG: Diacylglycerol

Figure 1. Diacylglycerol, Inositol-1,4,5-Triphosphate and
Protein Kinase C Second Messenger Pathway
[From K.D. Brown, J.Anim.Sci. 1987. 65
(Suppl. 2):140-156.]

Properties and Function of DG in Cell Signalling

The primary physiological role of DG is to activate membrane-bound PKC which then induces a series of physiological responses. The second messenger role of DG was first observed by Nishizuka and his colleagues (1984), who found that DG significantly increased the affinity of PKC for Ca^{++} and for phosphatidylserine (PS), thereby causing enzyme activation at the intracellular Ca^{++} level. The activation of PKC is believed to occur upon redistribution of the inactive cytosolic enzyme to the phospholipid environment of the membranes. The activation of PKC _in vivo_ may be a two-step process in which the enzyme initially binds, in a Ca^{++} - dependent manner, to cell membranes in an inactive but "primed" state (Bell, 1986). This primed enzyme is subsequently activated by the release of DG from the membrane phospholipid.

DG interacts with PKC at the same site as phorbol esters and is considered to represent the "endogenous phorbol ester". One of the phorbol esters, phorbol 12-myristate 13-acetate (PMA), is structurally similar to DG. Phorbol esters are more potent than DG in activating PKC, since they can insert themselves into the plasma membrane and are disposed of very slowly and activate their enzyme continuously. In contrast, both DG and IP3 exist transiently after stimulation and are rapidly degraded and are converted into a membrane phospholipid which restores the enzyme to a resting state

(Michell, 1983; Berridge, 1983). Studies with DG analogues established that the 3-hydroxyl group and both oxygen esters-of sn-1,2 DG are essential for activation. PKC is stereospecifically activated by the sn-1,2-DG configuration. DGs having a 2,3-sn configuration are not active in intact cells. These cell-permeable DGs provide direct evidence supporting a second messenger function for DG in a wide variety of cells (Bell, 1986).

DGs in the membranes disappear within a few seconds or at most several minutes of their formation. This rapid disappearance of DG is caused by its conversion back to inositol phospholipid by way of phosphatidic acid (PI turnover) and its further degradation to arachidonic acid, which can then be used for PG synthesis. In turn, PKC is active for a short period. However, the consequence of this enzyme activation may persist for a long period depending on the biological stability of the phosphate that is covalently attached to each substrate protein molecule (Nishizuka, 1986).

Function of IP3 in Cell Signalling

As described earlier, the hydrolysis of PIP2 yields two second messenger molecules, DG and IP3. DG molecules remain in the inner leaflet of the lipid bilayer in which they are able to move laterally by diffusion, and make contact with a membrane-bound enzyme, PKC; whereas IP3 is water soluble, and thus is able to diffuse away from the membrane into the

cytosol. IP3 acts on stored Ca^{++}; probably through its own receptor. However, IP3 once produced disappears rapidly, and a major mechanism for terminating this signal flow is considered to be removal of the 5-phosphate by the action of a specific phosphatase (Nishizuka, 1986).

The endoplasmic reticulum is a source of bound Ca^{++} which could be released by IP3. In addition, IP3 promotes the release of bound Ca^{++} from several microsomal preparations. A low concentration of the inositol polyphosphate induces rapid Ca^{++} mobilization from mitochondria. IP3 could activate a Ca^{++} conductance that allows efflux of Ca^{++} into the cytosol from the endoplasmic reticulum in exchange for K^{+} uptake. IP3 may bind to a specific receptor on the endoplasmic reticulum or calciosome causing opening of a specific calcium channel (Brown, 1987). Another interesting development in the inositol phosphate story is the discovery of a 1,3,4 isomer of inositol triphosphate (1,3,4-IP3), which is formed by the phosphorylation of IP3 to inositol 1,3,4,5-IP3 and subsequent dephosphorylation in the 5-position to give 1,3,4-IP3. IP3 may open calcium channels on the plasma membrane resulting in an influx of Ca^{++} from the extracelluar fluid (Kuno & Gardner, 1987; Gallacher, 1988). However, it is not known whether 1,3,4-IP3 is able to mobilize intracellular Ca^{++}. It is through the IP3 single system that the cell increases its intracellular free Ca^{++} concentration. It is suggested that the changes in ion concentration and pH may then stimulate the

transcriptional and protein synthetic events that lead to DNA synthesis (Brown, 1987).

PKC and Its Role in Cells

It has been found that PKC is present in all mammalian tissue and its activity often exceeds that of the cyclic AMP-dependent protein kinase A (PKA) (Nishizuka, 1984). PKC could play a central role in a wide variety of cell types. It has been proposed that PKC is involved with DNA synthesis, mRNA expression, cell growth and development, phosphorylation of receptor proteins in membrane and cytosol, as well as synthesis and release of peptide and steroid hormones. However, most of the studies on the PKC were conducted in tissues and cells other than those of the reproductive system.

Properties of PKC

PKC is a calcium-sensitive, phospholipid-dependent soluble protein kinase. PKC from rat brain cells is a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Mr ~82,000; indicating that the PKC is composed of one polypeptide chain (Kikkawa, 1982). PKC is further found to be composed of at least two functionally different domains. One is a hydrophillic domain that carries a catalytically active centre. These two domains can be separated by a Ca^{++}-dependent protease, calpain. The

isoelectric point of PKC is about 5.6. (Takai et al., 1985).
PKC is further characterized in that all forms of PKC (82 KDa)
are converted to two major fragments: a 45-49 KDa catalytic
fragment and a 36 KDa regulatory fragment. The cleavage of
these PKC subspecies by calpain I is enhanced by the presence
of phospholipid, DG or phorbol ester. Calpains I and II cleave
at one or two specific sites in the third variable region of
each PKC molecule (Kishimoto et al., 1989).

PKC Family

PKC is cleaved with the Ca^{++}-dependent neutral protease
(calpain I and II) to produce a catalytically active fragment.
Calpain I, which is active in the micromolar range of Ca^{++}, may
react preferentially with the active form of PKC that is
associated with membranes. Subsequently, PKC has been shown to
exist as a large family of multiple subspecies with subtle
individual differences (Mikawa, 1989). It has been found that
there are two distinct classes of phorbol ester (and DG)
receptor/protein kinase, conventional PKC (cPKC) and novel PKC
(nPKC). Each of these classes has multiple related molecules
expressed in tissues and cells in a type-specific manner. nPKC
may be involved in a variety of cell responses to
physiological stimuli and phorbol esters. It is possible that
nPKC in some cases is the major PKC activator. In addition,
functional diversity among cPKCs has been demonstrated: cPKC
gamma differs in its competence to mediate the signal toward

transcriptional activation through TPA-responsive acting elements from cPKC alpha and nPKC epsilon. The differences between cPKC and nPKC and among the individual members of each of these two classes, and their specific pattern of distribution in tissues and cells, should provide a rationale by which to explain the specificity and diversity of cellular responses to external stimuli generating DG (Ohno et al., 1991).

Seven isozymes of the PKC family have been reported in murine tumour cell lines. These isozymes are alpha, beta, gamma, delta, epsilon, zeta and eta. Some of the isozymes, PKC-alpha, -beta, and-eta, are differentially expressed in different lineages. PKC-alpha and -beta generally are not detectable in myeloid cell lines, where PKC-delta is the predominant isoform. Both PKC-alpha and -beta are abundant in most T and B lymphocytic lines (Mischak et al., 1991). It appears that different isozymes can play different roles. Activation of PKC causes enhancement of the responses of cAMP-generating systems in PC12 cells, which contain both alpha and gamma isozymes of PKC. IN NCB20 cells and NIH 3T3 cells, where only the alpha isozyme is expressed, activation of PKC causes inhibition of cAMP-generating systems. In NIH 3T3 cells after transfection of gamma-PKC, activation of the enzyme is no longer inhibitory; instead, there is a facilitation of cAMP accumulation (Gusovsky and Gutkind, 1991).

Substrate Proteins of Protein Kinase C

Activation of PKC can phosphorylate many proteins including cytosolic and cytoskeletal proteins. Nishizuka (1986) summarized these substrate proteins for PKC. A very intriguing finding was that PKC phosphorylates several receptor proteins including membrane proteins. The receptor proteins include insulin receptor, ß-adrenergic receptor, somatomedin C receptor and others. It has been found that PKC can phosphorylate the membrane proteins including Ca^{++}-transport ATPase, Na^+/K^+ ATPase, Na^+ channel protein, Na^+/H^+ exchange system, GTP-binding protein and others. The enzymes for PKC include cytochrome P450, DNA methylase, guanylate cyclase, tyrosine hydroxylase and others. The receptor protein for epidermal growth factor is phosphorylated by PKC at the site of a threonine residue, which lies just on the cytoplasmic side of the membrane. It is possible that one receptor can alter the activity of a second, heterologous receptor. Such receptor cross-talk could have important consequences for cellular regulation (Brown, 1987).

The substrate proteins for PKC are not known in detail in the CL, but there are some interesting findings. Maizels et al.,(1989) found that novel phosphorylation of a protein was stimulated by phospholipid and DG. This phospholipid/diolein-stimulated phosphorylation of a protein (80 KDa) was detectable in rat and porcine luteal extracts. One endogenous protein (37 KDa) appeared to serve as a preferred substrate

for phosphorylation by A kinase in SC and C kinase in LC in ovine CL. Another protein (81 KDa) was predominantly phosphorylated in large rather than small cells by a calcium-dependent, C kinase-independent mechanism (Hoyer and Kong, 1989). Furthermore, activation of PKC by PMA phosphorylated a calcium- and phospholipid-dependent endogenous protein in the membrane fraction prepared from bovine luteal cells. LH had a similar effect. LH may stimulate this endogenous protein (120 KDa to 18 KDa) phosphorylation via a PKC-mediated mechanism (Budnik and Mukhopadhyay, 1990).

Interaction between PKA and PKC Systems

Nishizuka (1986) described 'monodirectional' or 'bidirectional' control systems in which the two classes of receptors either potentiate one another or have opposing effects in cells. It appears that most tissues have at least two major receptor classes for transducing information from first messengers (hormones or others) across the cell membrane. One class involves the generation of cAMP second messengers, the other one depends on the production of DG, IP3, Ca^{++} mobilization, PKC activation, arachidonate release and cGMP formation. In bidirectional control systems, the classes of receptors appear to counteract each other, whereas in monodirectional control systems one receptor class may potentiate the other one (Nishizuka, 1986). Pinealocytes and

pituitary cells are examples of monodirectional cells in which PKC potentiates cAMP production. In contrast, in bidirectional cells such as erythrocytes and ovarian granulosa cells, PKC inhibits cAMP production. Small luteal cells in CL may behave as monodirectional cells, in so far as both second messenger systems stimulate P4 synthesis. Large luteal cells may act as bidirectional cells although no clear inhibiting effect of activating PKC on LH-stimulated P4 synthesis has been demonstrated (Hansel et al., 1986). However, activation of PKC decreased P4 production by LH-stimulated small ovine luteal cells (Wiltbank et al., 1989a). Mono- or bidirectional nature of cells should be further investigated.

More recently it has been found that activation of PKC could influence cAMP synthesis and secretion in the CL. PMA pretreatment increased the response of adenylate cyclase to a subsequent hormonal stimulation without changing the affinity of the receptors for the hormone in bovine luteal cells (Budnik and Mukhopadhyay, 1987). In swine mixed luteal cells, PMA had no effect on basal cAMP accumulation, but increased LH- and Forskolin-activated cAMP accumulation. DG mimicked the effect of PMA (Wheeler and Veldhuis, 1989). However, treatment of rat luteal cells with PMA or $PGF_{2\alpha}$ for 1 h produced a dose-dependent inhibition of LH-stimulated cAMP accumulation. PKC may be an important endogenous regulator of adenylate cyclase activity in porcine luteal cells (Rajkuma et al., 1991).

Hormonal Regulation of DG and IP3 Production

It has been found that LH, oxytocin, $PGF_{2\alpha}$, GnRH, insulin, neurotensin, TRH, vasopressin, acetylcholine and angiotensin can cause increases in inositol lipid turnover in different tissues (Brown, 1986). $PGF_{2\alpha}$ and LH initiate the breakdown of PIP2 to DG and IP3 in bovine luteal cells (Davis et al., 1987a b; Davis et al., 1988; Duncan and Davis, 1991). A similar effect of $PGF_{2\alpha}$ on DG and IP3 synthesis and secretion has been observed in the ovine (McCann et al., 1987), human (Devis et al., 1989) and rat (Leung et al., 1986; Lahav et al., 1988) luteal cells. In porcine ovarian cells, treatment with $PGF_{2\alpha}$ resulted in a translocation of phorbol dibutyrate binding from the cytosolic to the membrane-bound fraction, suggesting that $PGF_{2\alpha}$ activates PKC in ovarian cells (Veldhuis, 1987).

Function of PKC in Production of Peptide Hormones

Activation of PKC can increase synthesis and production of peptide and steroid hormones. It has been proposed that activation of PKC can increase synthesis and release of insulin, GnRH, growth hormone, prolactin, thyrotropin, catecholamine, parathyroid hormone and steroid hormones (Nishizuka, 1986). PMA is one of the phorbol esters and a strong activator of PKC. Treatment with PMA _in vitro_ for 2 h resulted in the increase of oxytocin secretion and P4 production in bovine mixed luteal cells. A23187 (a calcium

ionophore) also increased oxytocin secretion (Cosola-Smith et al., 1990). In the ovine, PMA and DG caused a dose-dependent stimulation of oxytocin secretion by luteal tissue slices in the presence of A23187. Phospholipase C also induced a dose-dependent stimulation of oxytocin secretion by ovine luteal slices (Hirst et al., 1990). PGs were observed to inhibit oxytocin release in the presence of IGF-I ($PGF_{2\alpha} > PGE_2 > PGE_1$) (McArdle and Holtorf, 1989). In pigs, PMA exerted a time- and dose-dependent stimulatory effect on relaxin release by enzyme-dispersed luteal cells in culture (Taylor and Clark, 1988) and DG mimicked the action of PMA in stimulating relaxin secretion.

PKC Activity and Its Effect in Steroidogenesis

PKC has been identified in a variety of exocrine and endocrine tissues including the CL of sheep (Wiltbank et al., 1989; Hoyer and Kong, 1989), cattle (Davis and Clark, 1983), pigs (Noland and Dimino, 1986; Wheeler et al., 1987) and humans (Clark et al., 1983). In the ovine, PKC activity was stimulated 2.9-fold in SC but not significantly enhanced above basal level in LC using PKC activators. PKC activators did not induce any differences in PKA activity between LC and SC. Maximal specific activities of both kinase were greater in SC than LC (Hoyer and Kong, 1989). Treatment with PMA for 15 min resulted in translocation of 91% of the available PKC to the membranes in ovine LC (Wiltbank et al., 1989). In pigs,

cytosolic fractions of CL contained 3 times more PKC-specific activity than did the cytosolic fraction of follicles. In contrast, mitochondria from medium follicles contained 30% more specific PKC activity than did luteal mitochondria (Noland and Dimino, 1986). The study of phorbol ester receptors suggested that the primary phorbol ester receptor in bovine luteal cells is PKC (Dowd et al., 1990). In addition, porcine luteal cytosol contains specific, high-affinity saturable, low-capacity [3H] PDB (a kind of phorbol ester) binding sites (Wheeler and Valdhuis, 1987).

Activation of PKC is reported to stimulate or inhibit P4 production depending on species, cell types and other experimental conditions. Phorbol esters were reported to stimulate P4 production by bovine small luteal cells (Brunswig et al., 1986; Alila et al., 1988). PMA potentiated the LH-stimulated cAMP accumulation and P4 production in the presence of low doses of LH. Paradoxically in the presence of maximal or minimal effective doses of LH, PMA exerted a time- and dose-dependent inhibition of P4 synthesis by bovine SC (Benhaim et al., 1987b). They did not report the results from LC. A23187 associated with PMA was able to mimic the stimulatory effect of phospholipase C on basal P4 production, whereas neither A23187 nor PMA alone had any effect in bovine SC (Benhaim et al., 1990).

In the ovine, both PMA and A23187 inhibited P4 accumulation in a dose dependent manner, but the two were not

additive in their effects; basal P4 accumulation was inhibited 30% by PMA and 10% by $PGF_{2\alpha}$ (Conley and Ford, 1989). Similarly, PMA inhibited P4 production by large ovine luteal cells and LH-stimulated SC (Hoyer and Marion, 1989). Wiltbank et al., (1989) reported similar results of PMA on P4 production in the ovine. $PGF_{2\alpha}$ is suggested to inhibit P4 production in ovine LC and SC by way of the PKC system (Wiltbank et al., 1990). Interestingly, the PKC system also may regulate steroidogenesis in placenta. Shemesh (1990) found that production of P4 in the placenta, especially the fetal cotyledon is uniquely cyclic-nucleotide independent, but Ca^{++} dependent, which indicates that the Ca^{++} second messenger and PKC systems are responsible for regulation of sterol biosynthesis in the cow placenta.

PKC and Steroidogenic Enzymes

The relationship between PKC and steroidogenic enzymes is poorly understood. $P450_{scc}$ activity, as measured by metabolism of 25-hydroxycholesterol, was inhibited by PMA in large but not in small ovine luteal cells, indicating that PKC acts before $P450_{scc}$ (Wiltbank et al., 1989). Bovine luteal cells were treated with the readily diffusible sterol, 25-hydroxycholesterol, but P4 production was not inhibited by $PGF_{2\alpha}$, suggesting that $PGF_{2\alpha}$ may exert its luteolytic effect at a site after cholesterol transport to the mitochondria but before $P450_{scc}$ (Grusenmeyer and Pate, 1992). However, the

investigation of gene expression for $P450_{scc}$ and 3ß-HSD provides another explanation. $PGF_{2\alpha}$ decreased levels of mRNA for 3ß-HSD indicating $PGF_{2\alpha}$ depresses the rate of synthesis and likely increases the rate of degradation of this messenger (Hawkins et al., 1992). In pigs, $PGF_{2\alpha}$ decreased basal P4 production and accumulation of mRNA for both $P450_{scc}$ and 3ß-HSD (Li et al., 1992). Activation of PKC therefore phosphorylates steroidogenic enzymes, but what kinds of enzymes are phosphorylated needs further investigation.

Ca^{++} and Steroidogenesis

As described earlier, IP3 can increase free calcium concentration from intracellular and extracelluar sources. Interestingly, hormones, such as $PGF_{2\alpha}$, LH and hCG cause an increase in free intracellular calcium concentration by bovine luteal cells (Davis et al., 1986, 1987a and b; Alila et al., 1989) or by rat ovarian cells (Leung et al., 1989). This section will especially discuss Ca^{++} and its role in P4 production in cattle and sheep.

Function of Ca^{++} on P4 Production by Bovine Luteal Cells

It has been found that large and small bovine luteal cells have a differential response to calcium with a remarkable parallelism between P4 production and intracellular

calcium levels. Large bovine luteal cells contain high levels of resting $[Ca^{2+}]$ but are relatively insensitive to LH. They require Ca^{++} for both basal and LH- and forskolin-stimulated P4 production (Alila et al., 1988a, 1989). Whereas small bovine luteal cells contain relatively low resting Ca^{++} concentrations and are extremely sensitive to LH, they are able to produce basal levels of P4 in the absence of calcium in the medium. Small bovine luteal cells require Ca^{++} ion for LH-, PGE_2-, 8-bromo-cAMP-, and $PGF_{2\alpha}$-stimulated P4 production (Hansel et al., 1991).

An inhibitor of intracellular calcium release and/or action in LC and SC, 8-N,N'-diethylaminocytyl-3,4,5-trimethoxybenzooate (TMB-8), inhibits both basal and LH-stimulated P4 production in large bovine luteal cells, but only LH-stimulated P4 production in the small cells. Addition of an inhibitor of calmodulin/PKC, N-(6-amiohexyl)-5-chloro-1-naphthalene sulphonamide (W-7) suppressed both basal and LH-stimulated P4 production in the LC and SC, indicating that calmodulin may be an intracellular regulator of basal P4 production, at least in the LC (Hansel et al., 1991).

Interestingly, addition of LH to small bovine luteal cells is followed immediately by a rapid transient rise in Ca^{++}, and a secondary elevation is observed and maintained for the duration of the experiment using a computer-driven spectrofluorometer (Alila et al., 1989). EGTA, a calcium chelator, abolished the secondary rise. This indicates that

the first phase is generally interpreted as being due to mobilization of intracellular calcium by IP3 and triggering of a calcium influx by 1,3,4,5-tetrakisphosphate. The secondary phase is a result of influx Ca^{++} from extracelluar sources. In contrast, only a single phase of Ca^{++} increase is observed in LH-treated LC and again this rise is abolished by addition of EGTA, suggesting that the LH-induced Ca^{++} rise is entirely dependent on an influx from an extracelluar source (Alila et al., 1989; Hansel et al., 1991).

Function of Ca^{++} on P4 Production by Ovine Luteal Cells

In sheep, A23187 causes a dramatic increase in free intracellular Ca^{++} in LC and SC. However, increased free Ca^{++} in the SC returned to control levels by 2 min after A23187, while Ca^{++} in the LC had not returned to baseline by 20 min after treatment. It seems that calcium extrusion mechanisms are more effective in the SC rather than in the LC (Wiltbank et al., 1991). Treatment with $PGF_{2\alpha}$ causes an increase in free intracellular Ca^{++} within LC but not SC. The free Ca^{++} in LC remains elevated for at least 30 min. This suggests that most of this increase in free intracellular Ca^{++} is due to influx from extracelluar sources. It is concluded that $PGF_{2\alpha}$ causes a sustained elevation in free Ca^{++} specifically in the LC (Wiltbank et al., 1991). The results indicate that large and small ovine luteal cells have clear functional differences, with $PGF_{2\alpha}$ responsiveness being more confined to LC which have

the high concentrations of $PGF_{2\alpha}$ receptors (Fitz et al., 1982; Balapure et al., 1989). In contrast to bovine luteal cells, LH does not cause an increase in free intracellular Ca^{++} in large and small ovine luteal cells (Wiltbank et al., 1989a).

In summary, calcium plays an important role in P4 production in large and small bovine and ovine luteal cells, and there is a species difference between sheep and cattle. Small but not large ovine luteal cells are extremely sensitive to LH, but LH does not cause an increase in free intracellular Ca^{++} in large and small ovine luteal cells (Wiltbank et al., 1989a). Small bovine luteal cells but not large cells are also extremely sensitive to LH but LH causes an increase in free intracellular calcium in both LC and SC. Small bovine luteal cells are more responsive to LH than LC in Ca^{++} and P4 production, even though their resting Ca^{++} level and basal P4 production rate is low (Hansel et al., 1991). $PGF_{2\alpha}$ causes an increase in free intracellular Ca^{++} in large and small luteal cells from the corpus luteum of sheep and cattle. The increase of Ca^{++} by $PGF_{2\alpha}$ is suggested to inhibit the ability of LH to stimulate P4 production in LC or to have a cytotoxic effect (Hansel et al., 1991).

MANUSCRIPT I

Responsiveness of Porcine Large and Small Luteal Cells to Luteotropic or Luteolytic Hormones and Cell Morphologic Changes During the Estrous Cycle and Pregnancy

ABSTRACT: Isolated porcine luteal cells from days 10 and 15 of the estrous cycle (estrus = day 0) were incubated with or without combinations of FSH (0, 10, 10^2, 10^3 ng), LH (0, 10, 10^3 ng), oxytocin or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (each at 0, 10, 10^3 and 10^5 pg). Progesterone (P4) content was determined after overnight incubation (0 h) then at 2 and 24 h of incubation. The basal (0 h) P4 production of large cells (LC) from day 10 CL was 31-fold higher than that by small cells (SC) at 0 h. The LC and SC from day 10, but not day 15, were stimulated to a small extent by LH ($P < 0.05$). The FSH inhibited P4 production ($P < 0.05$) by SC at 24 h on day 10 and by LC after 2 or 24 h of incubation on day 15. There was no interaction between LH and FSH on P4 production. Oxytocin and $PGF_{2\alpha}$ decreased P4 production by day 15 LC at 2 h of incubation ($P < 0.05$) and by day 15 SC after 2 or 24 h incubation ($P < 0.05$ and $P < 0.01$). The morphology of cells from corpora lutea (CL) of the cycle or early or mid-pregnancy was examined using scanning (SEM) and transmission (TEM) electron microscopy. Freshly isolated LC (using SEM) from day 10

contained many microvilli arranged in apparent networks on their membranes, but SC had smooth surfaces, and contained only a few microvilli. Internally, LC had more small mitochondria and a different organization of SER than did SC. The SC from pregnant CL (day 30 to day 60) contained more mitochondria than SC from cyclic CL. The results indicate that FSH, oxytocin, and $PGF_{2\alpha}$ can have a direct cellular luteolytic effect in the late luteal phase in pigs. The FSH influenced LC, whereas oxytocin and $PGF_{2\alpha}$ effected a more pronounced decrease in P4 from SC. The lower amount of P4 produced overall by SC may be associated with fewer microvilli, mitochondria, and SER.

Key Words: FSH, Oxytocin, $PGF_{2\alpha}$, Pigs, Corpus luteum, Morphology

Introduction

The role of putative luteotropic and luteolytic hormones in porcine luteal function has not been well defined. The influence of FSH on progesterone (P4) production was stimulatory for perfused bovine luteal cells (Romanoff, 1966) but inhibitory for porcine granulosa cells from large follicles (Hyeka and diZerega, 1990). Large luteal cells (LC), but not small luteal cells (SC), from cyclic gilts showed minimal response to LH (Buhr, 1987) whereas both LC and SC from pregnant sows demonstrated increased P4 secretion (Lemon and Loir, 1977).

The roles of oxytocin and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in porcine luteal function are likewise unclear. Although oxytocin may participate in luteolysis in vivo (Hansel et al., 1987) its direct effect on P4 production by luteal cells is controversial (Fuchs, 1988). Similarly, porcine CL are resistant to the luteolytic effects of $PGF_{2\alpha}$ in vivo during much of the cycle (Connor et al., 1976; Britt 1987) but any direct effects on luteal steroidogenesis are unclear.

The porcine SC produce much less P4 than do the LC (Yuan and Connor, 1990) and may respond differently to hormone challenge, but, the morphological differences between LC and SC of the cycle by scanning (SEM) and transmission (TEM) electron microscopy have not been studied in detail. The present investigations were designed to study 1) the effects of LH, FSH, oxytocin and $PGF_{2\alpha}$ on P4 production by porcine LC and SC during the mid (day 10) and late (day 15) luteal phase and 2) the morphological characteristics of porcine large and small luteal cells during the estrous cycle and early to mid-pregnancy using SEM and TEM. Mid-cycle (day 10) was chosen as approximating maximum CL development and P4 production in vivo but preceding the time of sensitivity to $PGF_{2\alpha}$ (Britt 1987). Morphological characteristics of CL from pregnancy, a time when luteotropic support is necessary, were used for comparison to those during the cycle.

Materials and Methods

Cell Isolation. CL were collected surgically from normally cyclic gilts on day 10 or day 15 of the estrous cycle (1st d of standing heat = day 0). Cell preparation was basically that described by Buhr (1987). In brief, the CL were put into chilled fresh media consisting of Ham's F-12 Nutrient Mixture (10.6 g/L, GIBCO, Grand Island, NY) containing 1.176 g of $NaHCO_3$, .014 g of polymyxin, .10 g of streptomycin, .002 g of insulin (Sigma Chemical, St. Louis, MO), .00004 g of hydro-cortisone, .005 g of transferrin (Sigma Chemical, St. Louis, MO), and .0025 g of metronidazole (Sigma Chemical, St. Louis, MO) per liter of deionized water. Aseptic techniques were employed and samples were kept on ice or at 4°C unless stated otherwise. The CL were decapsulated, weighed, chopped finely, and placed in 5 mL of media/g of tissue. Dissociation was carried out using sequential incubations (2 x 45 min; 1 x 30 min) in media containing 2 mg of collagenase (Type V, Sigma Chemical, St. Louis, MO) per ml of media (incubation media) at 37°C in a shaking water bath. Pellets formed from centrifugation of the retained supernatant were resuspended in the incubation media containing EGTA (1 mM, Sigma Chemical, St. Louis, MO) and incubated at 37°C a further 10 min. Final washing of the resulting cells with media was followed by layering onto a discontinuous Ficoll 400 (Pharmacia LKB, Sweden) gradient at room temperature. After 45 min, Ficoll layers with cells were filtered, pelleted (5

min, 500g) and washed four times with fresh media. The cells were counted, viability checked by 0.4% trypan blue exclusion, and cell numbers per milliliter of media were adjusted to 1 x 10^3 for large cells, and 10 x 10^3 for small cells. Cells were plated (24-well plate, Becton Dickinson, Lincoln Park, NJ) in 1 mL/well of fresh F-12 media containing 100 μg of low density lipoprotein and incubated at 37°C (95% air/5% CO_2, 100% humidity) overnight (12 to 16 h). Cell viability, using 0.4% trypan blue exclusion, was checked before and after incubation.

Low Density Lipoprotein (LDL). Lipoproteins were isolated from porcine serum collected on day 5 of the estrous cycle. Low density lipoproteins (LDL) were dialysed for 72 h in phosphate buffered saline as described by Buhr (1987). Protein concentration was determined according to Bradford (1976) and LDL were stored at 4°C for no more than 4 wk before use.

Experiment 1. To determine the responsiveness of LC and SC to FSH and LH, Exp. 1 was set up as a factorial applied to a completely randomized block design with four levels of FSH and three levels of LH applied in duplicate to wells of luteal cells from each pig (block). Luteal cells were obtained from pigs on day 10 (n = 4) or day 15 (n = 5) of the estrous cycle. After overnight incubation (12 to 16 h), incubation media were removed (0 h) and replaced with fresh media containing 100 μg of LDL and LH (0, 10, 10^3 ng; USDA pLH B-1, AFP-5400, NIH)

and(or) FSH (0, 10, 10^2, 10^3 ng; USDA pFSH-B-1, NIDDK-NHPP).
All incubations were done in duplicate. After 2 h, .5 mL of
medium was removed from each well and replaced with identical
fresh medium containing the appropriate hormone(s) for a
further 22 h at which time media were removed. Total
incubation time was 24 h. All media samples were stored at -
20°C until P4 was determined by RIA.

Experiment 2. Experiment 2 was also conducted as Exp. 1
with four levels of oxytocin (0, 10, 10^3, 10^5 pg) and the same
4 levels of $PGF_{2\alpha}$ to determine the effect of oxytocin (Sigma
Chemical, St. Louis, MO) and $PGF_{2\alpha}$ (Upjohn, Orangeville, ONT,
Canada) on P4 production by LC and SC. Luteal cells were
collected from pigs on day 10 (n = 4) or day 15 (n = 5) of the
estrous cycle. The procedures for incubation conditions,
medium removal and replacement were the same as those in Exp.
1.

Experiment 3. For SEM (Newbury et al., 1986), freshly
isolated luteal cells from cyclic pigs (day 10, n = 6 and day
15, n = 6) were used. These cells were washed with .1 M
cacodylate buffer (pH 7.4) in .015% $CaCl_2$ (4x, 500g). All the
procedures were carried out at 4°C. After resuspension the
cells were placed on poly-1-lysine (Sigma Chemical, St. Louis,
MO) covered round slides and fixed in 3% glutaraldehyde with
.1 M cacodylate, .015% $CaCl_2$, and 1% paraformaldehyde for 2 h.
The use of these chemicals can reduce shrinkage of cells
effectively (Newbury et al., 1986). They were postfixed in

.5% osmium tetroxide with .1 M cacodylate buffer. After dehydration in 10 to 100% ethanol alcohol gradient, the cells were transferred to 50/50 (vol/vol) acetone/ethanol alcohol, and then to 100% acetone. The cells were finally placed in liquid CO_2 for critical-point drying. The cells were examined by SEM (Cambridge Stereo Scan MKII A).

For TEM (Hunter, 1984), CL were surgically removed from cyclic sows (days 10, 15, 18, n = 6 each) or at slaughter from pregnant sows (day 30, n = 5 and day 60, n = 5). All fixatives were adjusted to pH 7.3 to 7.4 and chilled for 2 to 3 h before use. The tissues were fixed in .1 M cacodylate buffer and then in 1% osmium tetroxide in .1 M cacodylate buffer. The tissues were fixed in glutaraldehyde for 2 to 3 h, and postfixed in osmium tetroxide for 2 h. Thin sections (60 to 90 nm) were stained with 5% uranyl acetate in 50/50 (vol/vol) alcohol/acetone followed by lead citrate. Examination and photography were carried out with a Philips EM-200 electron microscope at magnifications from 1,000 to 40,000.

For SEM of fresh CL from pregnant pigs (day 30, n = 5 and day 60 n = 5), the CL were cut into small square pieces (2 mm^3) which were fixed in .1 M cacodylate buffer and then in 1% osmium tetroxide in .1 M cacodylate buffer. The rest of the procedures were done using the same methods as that in isolated cells by SEM. For both TEM and SEM, pictures were taken of 15 to 20 LC and 20 to 25 SC per animal. Both LC and

SC contained mitochondria cisternae and obvious SER cisternae typical of steroidogenic cells. The LC were considered to be those >25 μm and the SC were those < 20 μm.

Measurement of Progesterone. The concentration of P4 in media was determined by (RIA) (Yuthasastrakosol et al., 1974) without ether extraction using P4 antisera developed in rabbits (A18, N. Rawlings, University of Saskatchewan). The inter- and intraassay CV were 12.3 and 5.7%, respectively. The mean sensitivity of the assay at 95% binding was 12 pg/tube. Progesterone concentrations were expressed as pg P4$\cdot$10^3 cells$^{-1}\cdot$h^{-1}.

Statistical Analysis. In Exp. 1, FSH at 0, 10, 10^2, 10^3 ng/well and LH at 0, 10, 10^3 ng/well were used in a 4 x 3 factorial arrangement of treatment applied to a randomized complete block design. In Exp. 2, oxytocin and PGF$_{2\alpha}$ each at 0, 10, 10^3, 10^5 pg/well were used in a 4 x 4 factorial arrangement, again applied to a randomized complete block design. In both experiments, blocks consisted of individual pigs, four blocks in Exp. 1 and 5 in Exp. 2. The model for evaluating the effects of LH and FSH on P4 production was: $Y_{ijkl} = U + L_i + F_j + LF_{ij} + A_k + LFA_{ijk} + E_{ijkl}$ where Y_{ijkl} is P4 concentration observed for the i'th level of LH, j'th level of FSH, for the l'th cell culture drawn from k'th pig. LF_{ij} is the interaction between LH and FSH. LFA_{ijk} is the interaction of LH, FSH and pig. The error term used to test treatment effects was the treatment by block (i.e. pig) interaction with

LH and FSH (LFA$_{ijk}$). The CV for the variation between duplicate wells was 1%, 1% and 6% at 0, 2 and 24 h respectively. The model used for oxytocin and PGF$_{2\alpha}$ was similar to that for LH and FSH. Data were log-transformed to stabilize the variance and analyzed by the GLM procedure (SAS, 1985). Treatment effects were evaluated for each cell type at each incubation time of each day. Differences were tested using the Bonferroni test (SAS, 1985).

Results

Experiment 1. The cell isolation procedure produced relatively pure small-cell preparations, the contamination with LC being 0.42±0.06% (x±sem), whereas the contamination of LC by SC was 15±2.94% (x±sem). Viability of LC and SC at plating was 70 to 85% after isolation and 68 to 71% after 24 h incubation.

Basal P4 production (0 h) of LC from day 10 CL exceeded that of day 15 (3732 ± 117 vs 1094 ± 66 pg·10^3 cells^{-1}·h^{-1}, day 10 and day 15, respectively, P < 0.05), whereas SC P4 production was similar on days 10 and 15 (122 ± 13 vs 101 ± 3 pg·10^3 cells^{-1}·h^{-1}). The results of incubation with LH, FSH, oxytocin or PGF$_{2\alpha}$ on P4 production are shown in Figures 1 and 2.

No FSH x LH interactions on P4 secretion were noted, so the effects are presented independently. Progesterone production by day 10 cells was not affected by incubation with

LH for 2 h; however, after 24 h, the highest dose of LH (10^3 ng) increased P4 production by day 10 LC and SC. (Figure 1, C; Figure 2, C; $P < .05$). Day 15 LC and SC were not stimulated by LH. The FSH did not influence LC P4 production on day 10 but the higher doses of FSH decreased P4 secretion from day 15 large cells. The decline in P4 was significant after 2 h with 10^3 ng, and after 24 h with 10^2 and 10^3 ng of FSH ($P < .05$, Figure 1, B, D). The P4 production by SC was unaffected by 2 h incubation with FSH on day 10 (Figure 2, A) but declined after 24 h incubation with 10^3 ng of FSH (Figure 2, C; $P < .05$). The FSH was without effect on day 15 SC.

Experiment 2. There was no interaction between oxytocin and $PGF_{2\alpha}$, so the effects are presented independently. Neither oxytocin nor $PGF_{2\alpha}$ induced any significant change in P4 secretion by LC or SC on day 10. However, both oxytocin and $PGF_{2\alpha}$ decreased P4 secretion by SC of day 15. The P4 secretion by SC was decreased by 10^3 pg of oxytocin at 2 h ($P < .05$, Figure 2, B) and by all concentrations of oxytocin at 24 h of incubation ($P < .05$, Figure 2, D). The $PGF_{2\alpha}$ at all dose levels depressed P4 production by SC at 2 h and 24 h ($P < .05$ and $P < .01$, Figure 2, B, D). Oxytocin and $PGF_{2\alpha}$ at 10^3 pg decreased P4 production by d-15 LC at 2 h ($P < .05$; Figure 1, B), but this inhibition disappeared after 24 h incubation ($P > .05$; Figure 1, D).

Experiment 3. Examination of luteal cells from cyclic pigs by SEM showed that fresh mid-cycle LC (> 25 μm) were

round, regular, and had numerous microvilli and long
processes. Villi of varying lengths were erect or lay on the
surface (Figure 3, A) frequently forming networks with other
villi (micronetworks) (Figure 3, C). Small, deep depressions
were numerous. Fresh LC from day 15 CL appeared shrunken
(Figure 3, D). Day 10 SC (< 20 μm) were round with smoother
surfaces (Figure 3, B). The TEM revealed that LC membranes
were convoluted, with many microvilli and long processes.
Numerous small mitochondria were clustered around the
periphery of the SER and throughout the cytoplasm. There were
two different kinds of LC: one contained tiny, small, or non-
tubular-shaped SER (Figure 4, A); the other, approximating 35%
of the LC, had large, fingerprint tubular-shaped SER (Figure
4, B). The SC had vesicles of SER, but not large fingerprint
SER, more lipid droplets than LC and mitochondria. Membranes
were much more regular and contained a few microvilli (Figure
5, A). The LC of day 15 seemed to be undergoing regression.
Almost all day 15 LC appeared to have shrunken, but they still
had numbers of small mitochondria and prominent cellular
processes. Fingerprint SER, however, was not evident in LC
(Figure 6, A). As well, mitochondria, and lipid droplets had
almost disappeared in SC. By day 18, very few intact cells
were apparent (Figure 6, B).

The SEM from slices of pregnant CL (days 30 and 60)
demonstrated that there were many small and large cells
present (Figure 3, E). The LC contained numerous microvilli,
which also consisted of micronetworks (Figure 3, F). Many

characteristics of SC in the pregnant CL by TEM were similar to those of the mid-cycle; i.e. smooth surfaces and many vesicles of SER. However, the SC from pregnant CL (Figure 5, B) contained more mitochondria than those from cyclic CL (Figure 5, A). The nuclei were also different. The nuclei were leaf-shaped in pregnant SC (Figure 5, B), but round in cyclic SC (Figure 5, A). The LC contained many mitochondria and large fingerprint SER. Approximately 53% of the LC on day 30 and 66% on day 60 of pregnancy contained large fingerprint SER. The membranes were convoluted with many microvilli and long processes.

Discussion

In the present study, LH approximating physiological doses (10 ng·ml^{-1}) and above were used to study its effect on porcine LC and SC. The LH had very little effect on P4 production by either cell type, which is consistent with the findings of Buhr (1987) and Agu (1990) who used a similar system. The LC and SC from pregnant sows, however, were stimulated by pLH (Lemon and Loir, 1977). In other studies using mixed porcine luteal cells, LH had either a minimal (Hunter, 1981) or strong (Mattioli et al., 1985; Li et al., 1991) stimulation. Mixed luteal cell cultures may afford important communication between SC and LC that is essential for optimal P4 production and responsiveness to LH. This has not yet been elucidated. The bovine and ovine SC are more

responsive to added LH than LC (Fitz et al., 1982; Hansel et al., 1987), which is consistent with the majority of LH receptors being found on SC (Fitz et al., 1982). Porcine luteal LH receptors have also been quantified (Ziecik et al., 1980; Rao and Edgerton, 1984). The LH receptor concentration increased between days 6 and 10 of the cycle and dropped between days 12 and 14 (Ziecik et al., 1980). However, because dispersed or homogenized cells were used, it is not known which size of cell contains the majority of LH receptors.

Since porcine luteal cells are not consistently sensitive to LH (Hunter, 1981; Buhr, 1987; Yuan and Connor, 1990), some researchers regard the porcine CL as being autonomous (Hunter, 1981; Buhr, 1987). Recently, the "constitutive" induction of $P450_{scc}$ was proposed to explain the failure of LH to stimulate luteal cells early in the cycle (Hansel et al., 1991). This failure may result from high amount of mRNA for $P450_{scc}$ in luteinized granulosa cells suggesting these cells are already producing P4 at a maximal level (Oonk, 1989; Hansel et al., 1991; Aflalo et al., 1992; Hansel, personal communication, 1992). However, data from different laboratories may differ for various reasons including experimental design, stage of cycle cells were collected, cell numbers, cell type, media used, and incubation time.

The role of FSH in luteal P4 production remains elusive. The FSH stimulated P4 production by rat (Adashi et al., 1981)

and pig granulosa cells (Barano and Hammond, 1985) and by perfused bovine luteal cells (Romanoff, 1966). However, FSH alone was without effect on luteal tissue weight, P4 content and concentration when administered on day 7 of the bovine estrous cycle (Hansel and Seifart, 1976). The FSH receptors were present in bovine luteal cells, and interestingly, increased FSH receptors were noted in the early and late luteal phase (Manns et al., 1984). Ziecik et al (1988) did not find specific FSH receptors in luteal tissue from two pigs at unspecified days of the mid-luteal phase, but tissue from very early or later in the cycle were not analyzed. In the present study, FSH was originally hypothesized to have a possible synergistic function with LH, because FSH has a high basal level during the estrous cycle (Rayford et al., 1974) and has a demonstrated steroidogenic effect in other species (Romanoff, 1966; Adashi et al., 1981; Barano and Hammond, 1985). Surprisingly, in the present study FSH did not display any luteotropic function in porcine isolated luteal cells. Rather it decreased P4 production by LC of day 15 in a dose dependent manner. The FSH has also been found to inhibit P4 production by porcine granulosa cells from large follicles (Hylka and diZerega, 1990). The inhibitory effect of FSH on P4 production may benefit early follicular development.

Oxytocin and $PGF_{2\alpha}$ have both been considered as luteolysins. Oxytocin has been implicated in luteolysis in sheep, goats, cows, and sows (Sheldrick et al., 1980; Schams

et al., 1983; Cooke and Homeida, 1985; Hansel and Dowd, 1986; Fuchs, 1988). However, results from in vitro experiments have been conflicting. Oxytocin at a low concentration stimulated bovine luteal cells to secrete P4, but inhibited gonadotropin-induced P4 secretion at higher doses (Tan et al., 1982; Tan and Biggs, 1984). Other studies suggested that oxytocin had little direct effect on P4 production by rat, human and bovine luteal cells (Mukhopadhyay et al., 1984; Richardson and Masson, 1985; Hansel and Dowd, 1986). As well, oxytocin given in the early luteal phase suppressed PGI-2 biosynthesis by bovine luteal cells very substantially, suggesting that the luteolytic effect of oxytocin in the cow may be mediated by inhibiting the synthesis of this luteotrophic agent within the CL (Hansel and Dowd, 1986). However, mixed cell types were used and it was not known upon which cells oxytocin may have been acting. In the present study, oxytocin, although not luteolytic in day 10 cells, decreased P4 secretion by day 15 LC and SC at 2 h incubation and by SC at 24 h. Generally, day 15 cells produced much less P4 than did day 10 cells. Along with morphological differences, this suggests that luteolysis had already begun. Oxytocin may have enhanced a process already underway at the time of ovariectomy on day 15. The inhibitory effect of oxytocin on P4 production has also been noted in mixed, dispersed porcine luteal cells from day 13 of the estrous cycle (Przala et al., 1986). Oxytocin, which is an endogenous component of porcine CL (Pitzel et al., 1984),

may be involved in natural luteolysis. In any event, the results all indicate that porcine luteal cells become sensitive to luteolytic hormones in the late luteal phase.

The $PGF_{2\alpha}$ has been shown to enhance basic or LH-stimulated P4 production in dispersed porcine luteal cells from the mid- to late luteal period (Mattioli et al., 1985) and from early pregnancy (Wiesak, 1991). In the current work, on day 15, $PGF_{2\alpha}$ at 10^3 pg decreased P4 production by LC after 2 h but not 24 h incubation. The $PGF_{2\alpha}$ at all doses inhibited P4 production by day 15 SC throughout the incubation period. Our observation of the strong inhibition of P4 production in day 15 SC may provide a unique insight into the process of luteolysis. Luteolysis in the late luteal phase may be initiated in the SC. The SC only were sensitive to perturbants of membrane phospholipid methylation (Agu, 1990), which is consistent with altered membrane fluidity associated with luteolysis in other species (Buhr et al., 1979; Carlson et al., 1982; Sawada and Carlson, 1991). This concept of a differential luteolysis between LC and SC is further supported by our observations of morphological dissimilarities on day 15. Mitochondria and lipid droplets were still obvious in LC but had almost disappeared in SC suggesting a more advanced stage of luteolysis. Basal P4 production was lower in day 15 luteal cells implying in vivo agents had already begun to influence steroidogenesis. Although Gadsby et al. (1990) characterized high-affinity $PGF_{2\alpha}$-binding sites on porcine LC

from day 13, their work does not preclude the possibility that SC capable of low-affinity $PGF_{2\alpha}$-binding at day 13 develop high-affinity binding sites by day 15 or can be responsive to high $PGF_{2\alpha}$ concentrations. If maximum P4 production by LC is dependent upon some factor(s) from SC as seems to be the case in porcine CL of mid-pregnancy (Lemon and Mauleon, 1982) then initial luteolysis in the SC would be a logical prelude to CL regression.

The present results of TEM demonstrated that the number of mitochondria increase significantly in SC from pregnancy (Yuan and Connor, 1990b) compared to the estrous cycle. In the bovine, small luteal cells from the cycle had few mitochondria (Hansel and Dowd, 1986). Interestingly, porcine luteal LH receptors increased significantly during pregnancy (day 30, Ziecik et al., 1980), but it was not known on which cell type. The LH may regulate differentiation of SC to LC, a suggestion originally presented by Donaldson and Hansel (1965). This proposal was demonstrated in the experiment (Alila and Hansel, 1984) in which some SC (thecal origin) apparently transformed into LC after the 6th day of the estrous cycle in the bovine. During early pregnancy, LC (granulosa origin) disappeared, but the cells of the thecal origin persisted throughout pregnancy. In ovine and porcine CL (Farin et al., 1988; Wiesak, 1989), the numbers of LC increased, and the numbers of SC decreased after injection of hCG during the luteal phase. Although the secretion of P4 by

LC may not be regulated directly by LH, the number of LC may depend, at least in part, on this hormone (Niswender and Nett, 1988). The increased mitochondria in porcine SC of pregnancy may be related to high blood LH associated with high levels of LH in the pituitary gland during early pregnancy (Parlow et al., 1964; Melampy et al., 1966), but it is not clear if SC with increased mitochondria can develop into LC.

Belt et al. (1970) noted that SER cisternae in parallel array were present in LC from CL of pregnant sows, but were rarely found in CL from nonpregnant sows. However, numerous SER cisternae in parallel array were present in LC from the cyclic CL in our study. Alpha and beta LC have been reported in bovine CL (Fields et al., 1991). Beta LC contained numerous mitochondria and a more apparent SER than small alpha LC; beta LC were more sensitive to $PGF_{2\alpha}$ and underwent regression first (Fields et al., 1991). Two types of LC in the present work also could be divided into alpha LC and beta LC due to their different SER. Alpha LC contained tiny, small or non-tubular-shaped SER, but beta LC had large fingerprint, tubular-shaped SER. This also could contribute to the difference in responsiveness to $PGF_{2\alpha}$ noted in this and other studies that could be dependent on purity of isolated cell fractions. As well, with individual animal variabilities in estrous cycle lengths more or fewer beta-type LC could be present and responsiveness to $PGF_{2\alpha}$ be reflected accordingly.

Microvilli of LC from cyclic and pregnant CL were

similar; they were also similar to those from granulosa cells in porcine follicles (Miyamoto et al., 1982). Microvilli on the surface of LC consisted of many micronetworks, which may provide a direct passage for rapid communication and material transportation important to autocrine and paracrine functions. Compared with LC, SC in cyclic CL contained fewer microvilli, fewer SER and fewer mitochondria. Basically, LC produced approximately 10 to 30-fold more P4 than SC on day 10 in our work. In the ovine, LC produced 47 times more P4 than SC under basal conditions (Wiltbank et al., 1991). Because mitochondria and SER contain many key enzymes for P4 synthesis (Robert and Armstrong, 1988), and microvilli on the cell surface enlarge the absorption and secretory surface area of the cells, fewer microvilli, mitochondria, and SER may be directly responsible for the lower capacity of SC in P4 production compared with LC. Although these results demonstrated that porcine luteal cells do have some characteristics in common with the CL of the ruminants, we also noted differences. The primary distinguishing feature was the fingerprint and tubular SER seen in porcine LC. This abundance and arrangement of SER is not seen in the ruminant LC but is confined to the SC (Hansel et al., 1987).

In summary, oxytocin and $PGF_{2\alpha}$ inhibited day 15 SC production of P4. The FSH inhibited P4 secretion by day 10 SC and by day 15 LC. Incubation of cells with LH had little effect on P4 secretion. These results support the luteolytic

potential for oxytocin, $PGF_{2\alpha}$ and FSH during the latter part of the porcine luteal phase. This unique effect of FSH on pig luteal cells, which may be self-serving for enhanced follicular development, deserves closer examination. Luteolysis seems to proceed by different mechanisms and perhaps at slightly different time intervals in SC than in LC. Unlike SC, the LC contained many microvilli and micronetworks on the plasma membrane along with numerous mitochondria and SER. The lower P4 production by SC may be associated with these different morphological features. The increased number of mitochondria in SC of pregnancy may be associated with their development into LC or with increased involvement with LC P4 production.

Figure 1. The effects of LH, FSH, oxytocin or $PGF_{2\alpha}$ on progesterone production (pg.10^3 cells^{-1}.h^{-1}, LSM ± sem) by the large luteal cells from days 10 (n = 4 pigs) or 15 (n = 5 pigs) of the estrous cycle. Incubation with or without LH, FSH, oxytocin and $PGF_{2\alpha}$ were done in duplicate with cell types from corpora luteum from each pig. Progesterone production by the large cells is shown after A) 2 h incubation on day 10; B) 2 h incubation on day 15; C) 24 h incubation on day 10; D) 24 h incubation on day 15. ☆ different from 0 level (P < 0.05).

Figure 2. The effects of LH, FSH, oxytocin or $PGF_{2\alpha}$ on progesterone production (pg.10^3 cells^{-1}.h^{-1}, LSM ± sem) by the small luteal cells from days 10 (n = 4 pigs) or 15 (n = 5 pigs) of the estrous cycle. Incubation with or without LH, FSH, oxytocin and $PGF_{2\alpha}$ were done in duplicate with cell types from corpora luteum from each pig. Progesterone production by the large cells is shown after A) 2 h incubation on day 10; B) 2 h incubation on day 15; C) 24 h incubation on day 10; D) 24 h incubation on day 15. ☆ difference from 0 level (P < 0.05), ☆☆ different from 0 level (P < .01).
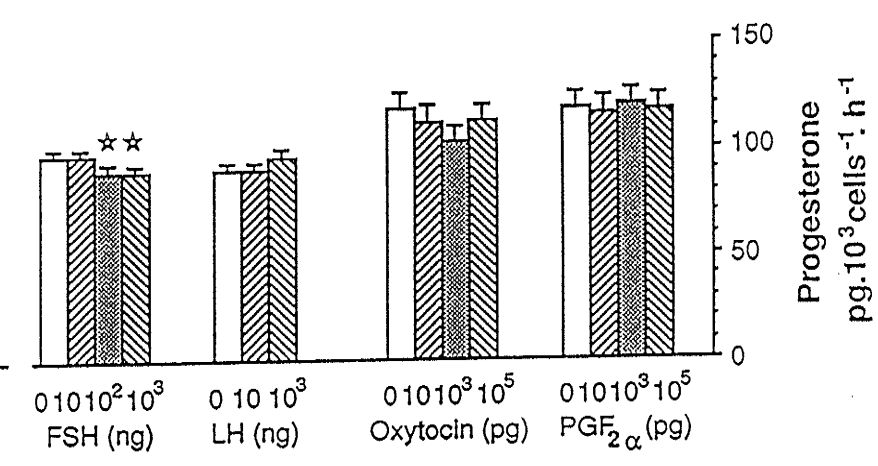
A 2h Large Cells Day 10
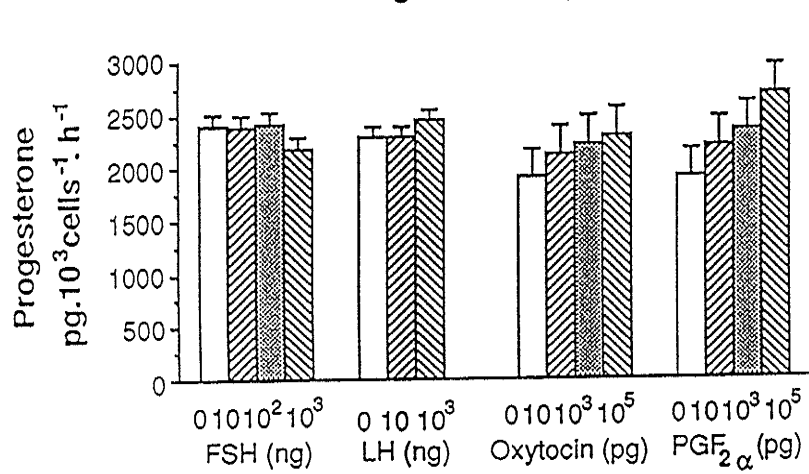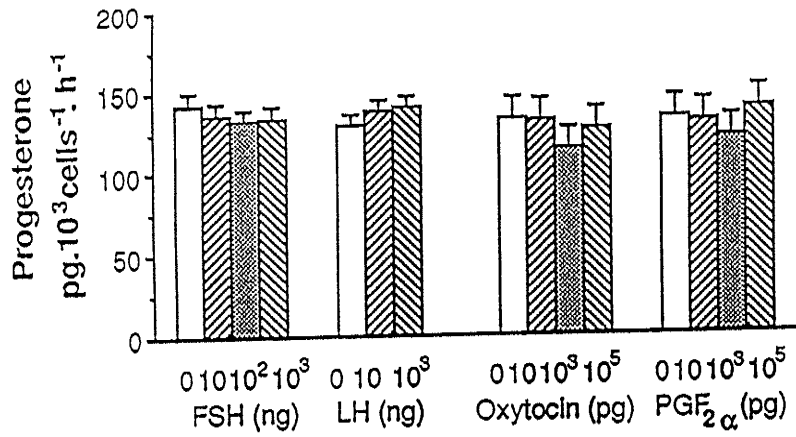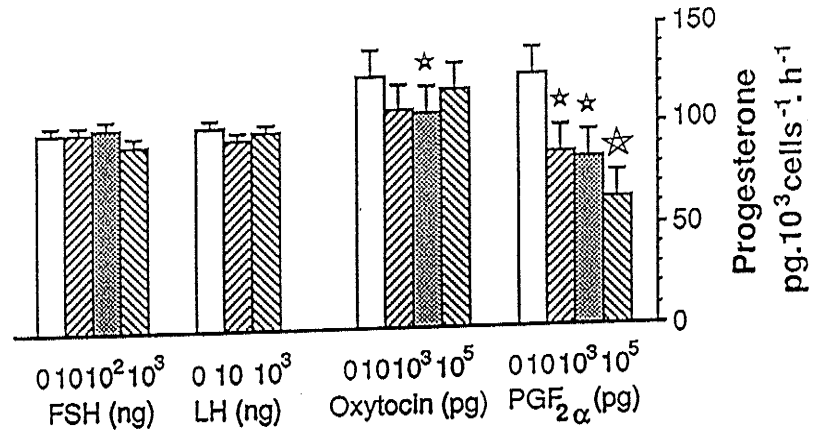
B 2h Large Cells Day 15
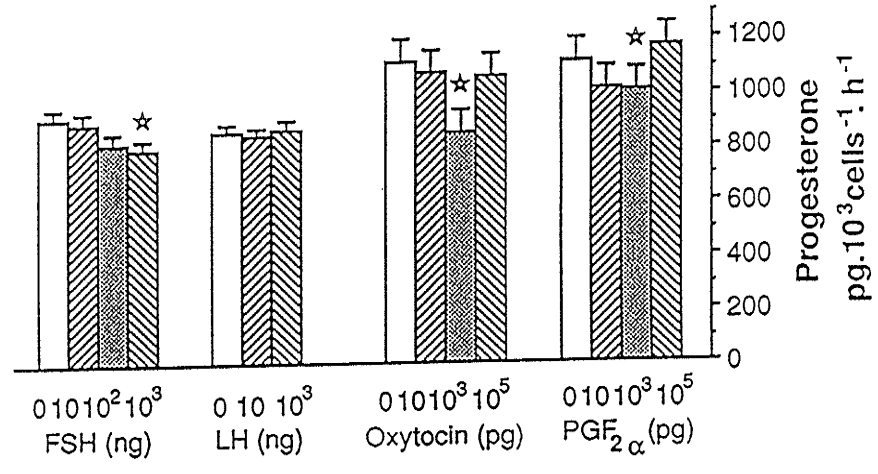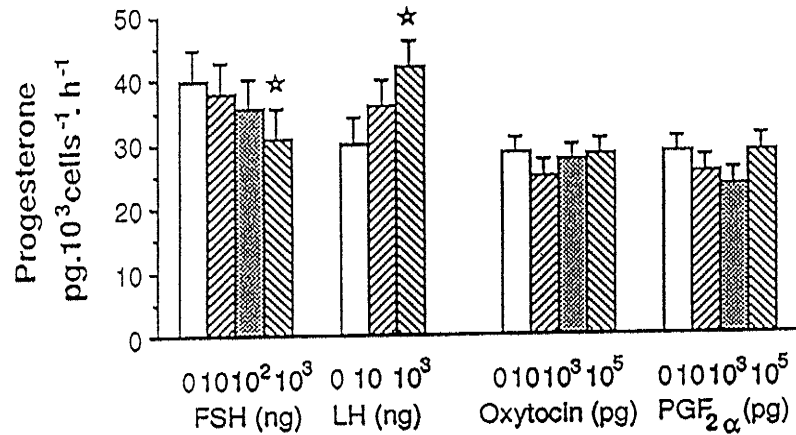
C 24h Large Cells Day 10
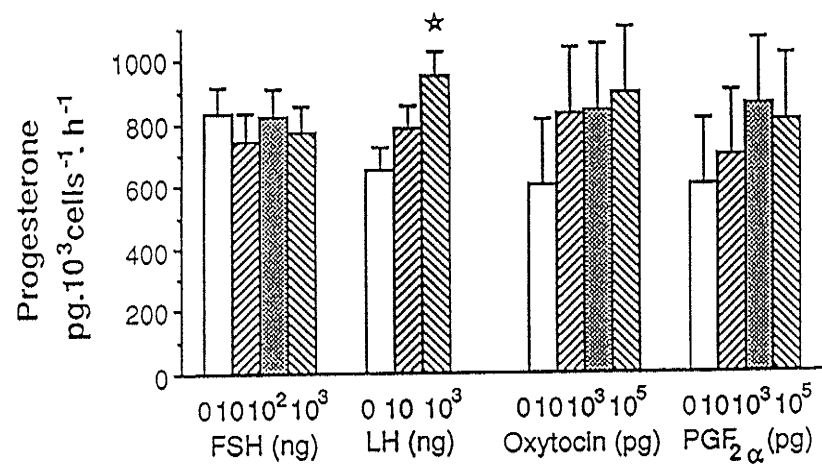
D 24h Large Cells Day 15

A 2h Small Cells Day 10

B 2h Small Cells Day 15

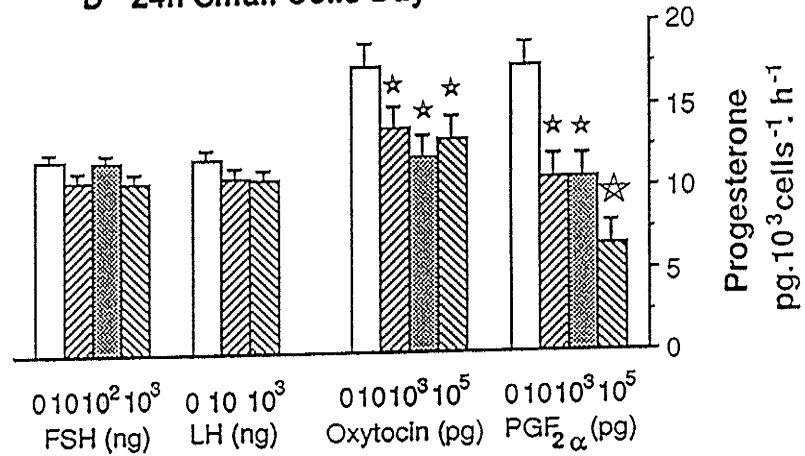**C 24h Small Cells Day 10**

D 24h Small Cells Day 15

Figure 3. Scanning electron microscopy of porcine luteal large and small cells during the estrous cycle and pregnancy.

A.   An isolated large cell (38 μm) with many microvilli on its membrane from a corpus luteum of day 10 during the estrous cycle. X2600.

B.   An isolated small cell (17 μm) with smooth surface from a corpus luteum of day 10 during the estrous cycle. X2600.

C.   The membrane surface with many microvilli and microvilli arranged in micronetworks in an isolated large cell from day 10 of the estrous cycle. X5200.

D.   Two shrunken large cells (both 34 μm) from a corpus luteum of day 15 during the estrous cycle. X1300.

E.   Large and small cells in a corpus luteum of day 30 during the pregnancy. X500.

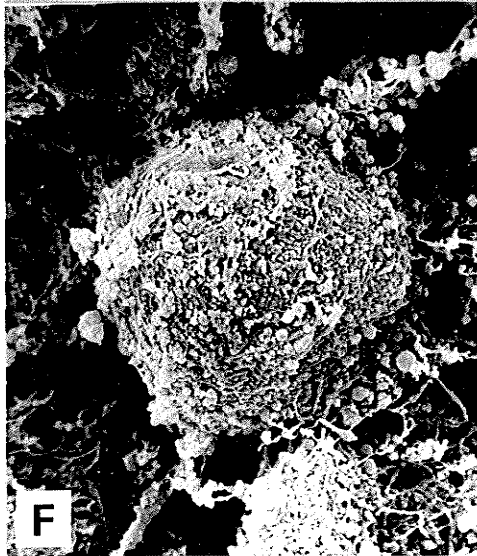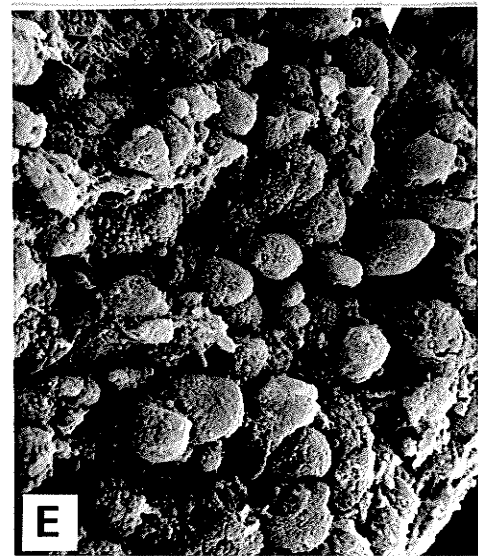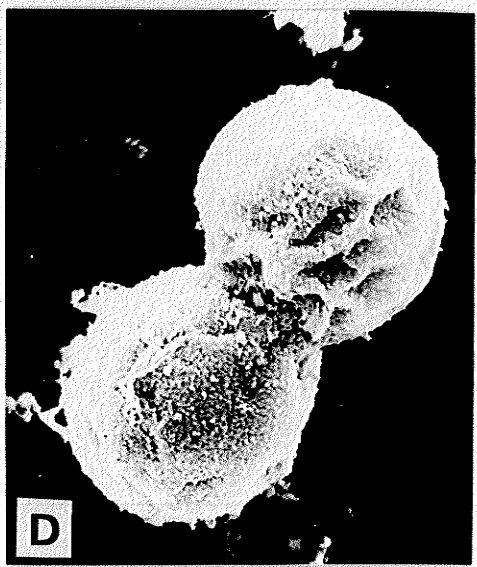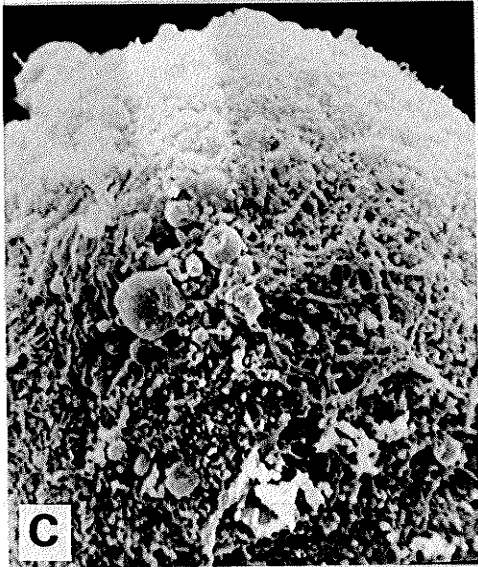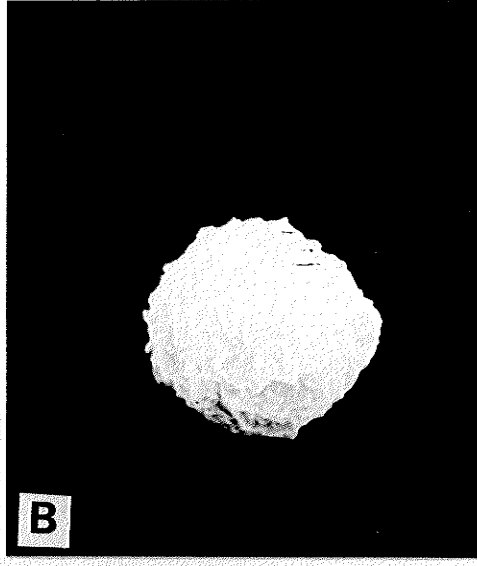F.   A large cell (30 μm) from a corpus luteum of day 60 during pregnancy. X2200.

Figure 4. Transmission electron microscopy of porcine large
luteal cells during the estrous cycle.

A.   A large cell from a corpus luteum of day 10 during the
estrous cycle. The cell contained numerous tiny and
small or short-tubular shaped SER, but not large,
fingerprint tubular-shaped SER. X2500.

B.   A large cell from a corpus luteum of day 10 during the
estrous cycle. The cell contained large fingerprint
tubular-shaped SER that occupied approximately 40% of the
cell area. X3000.

Figure 5. Transmission electron microscopy of porcine small luteal cells during the estrous cycle and pregnancy.

A.   A small cell (sc ,17 μm) from a corpus luteum of day 10 during the estrous cycle. Arrow indicates the large fingerprint SER in a large cell.

B.   A small cell (14 μm) from a corpus luteum of day 60 during the pregnancy. Note: The number of mitochondria increased within the cytosol. X6000.
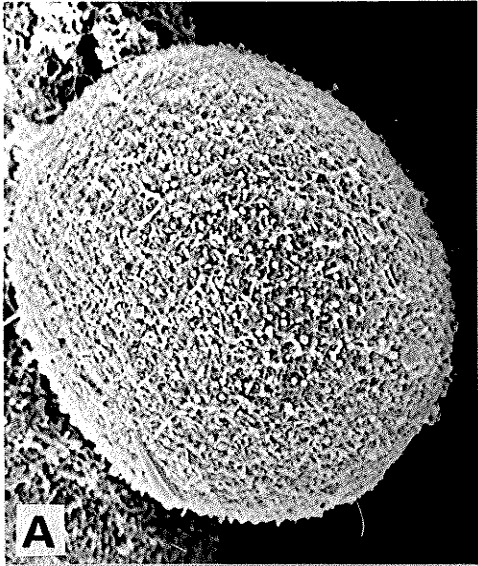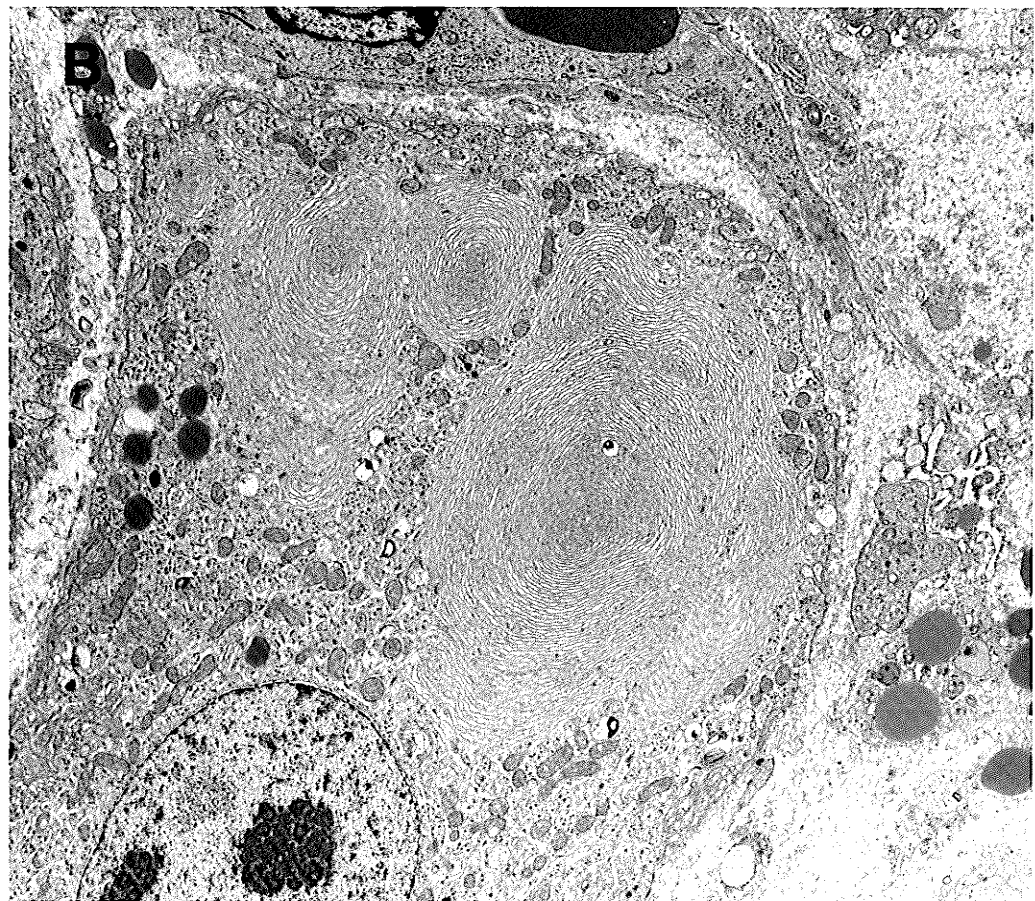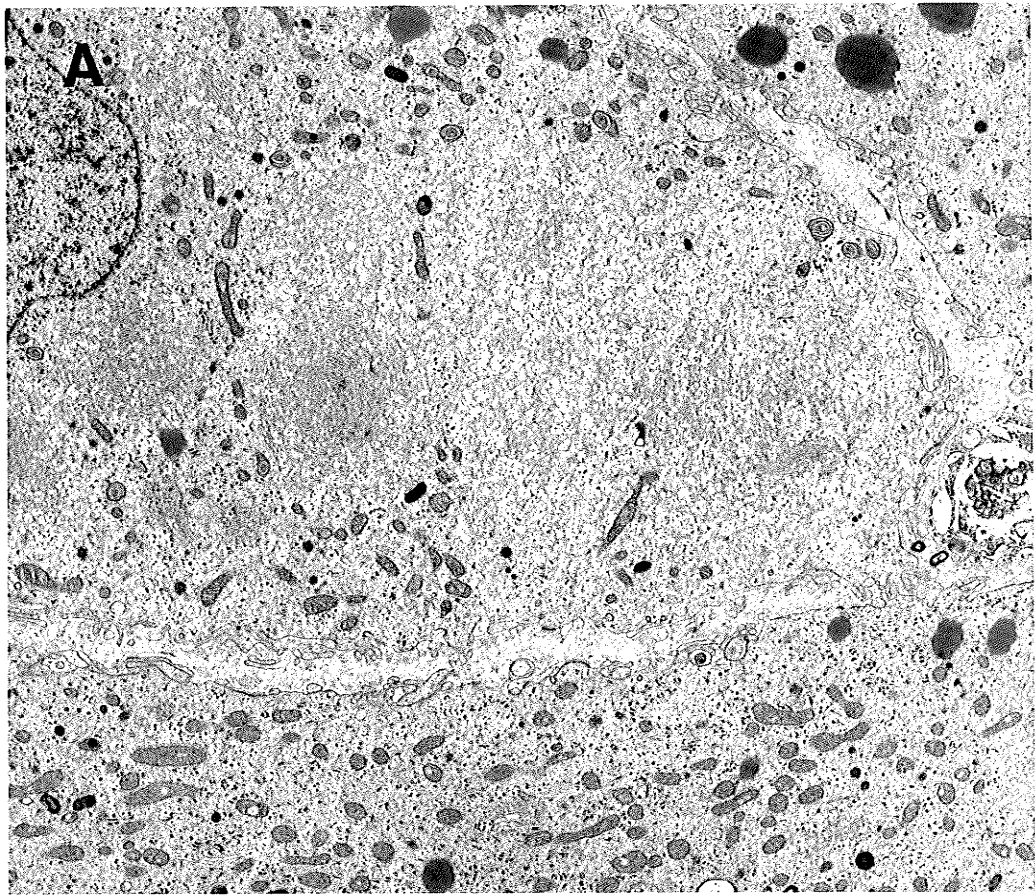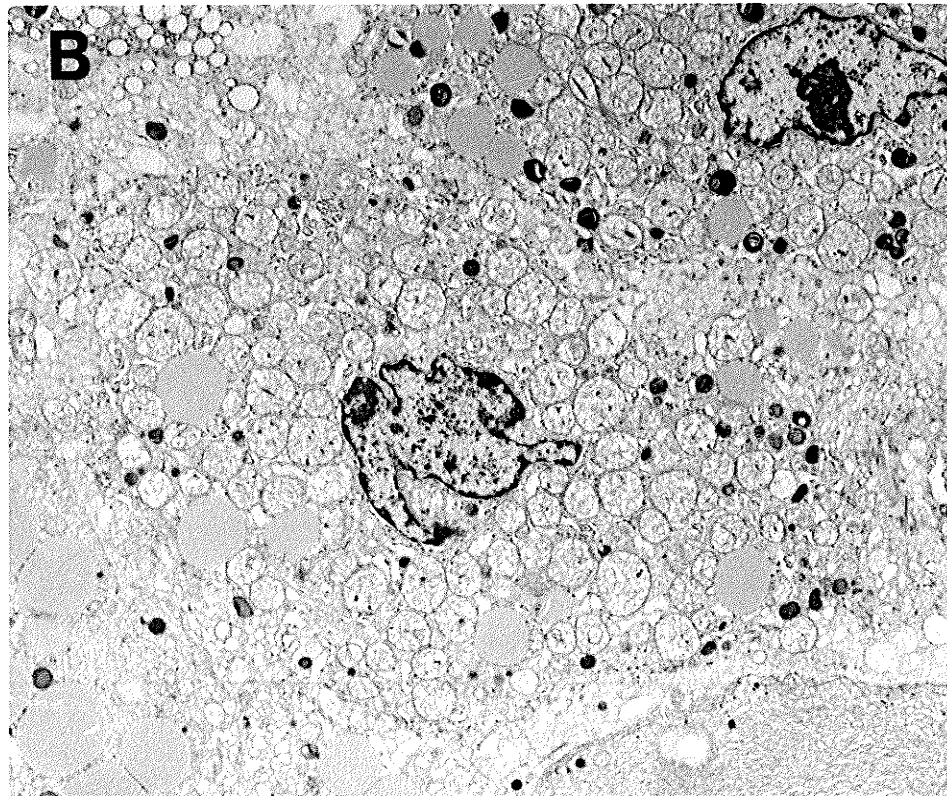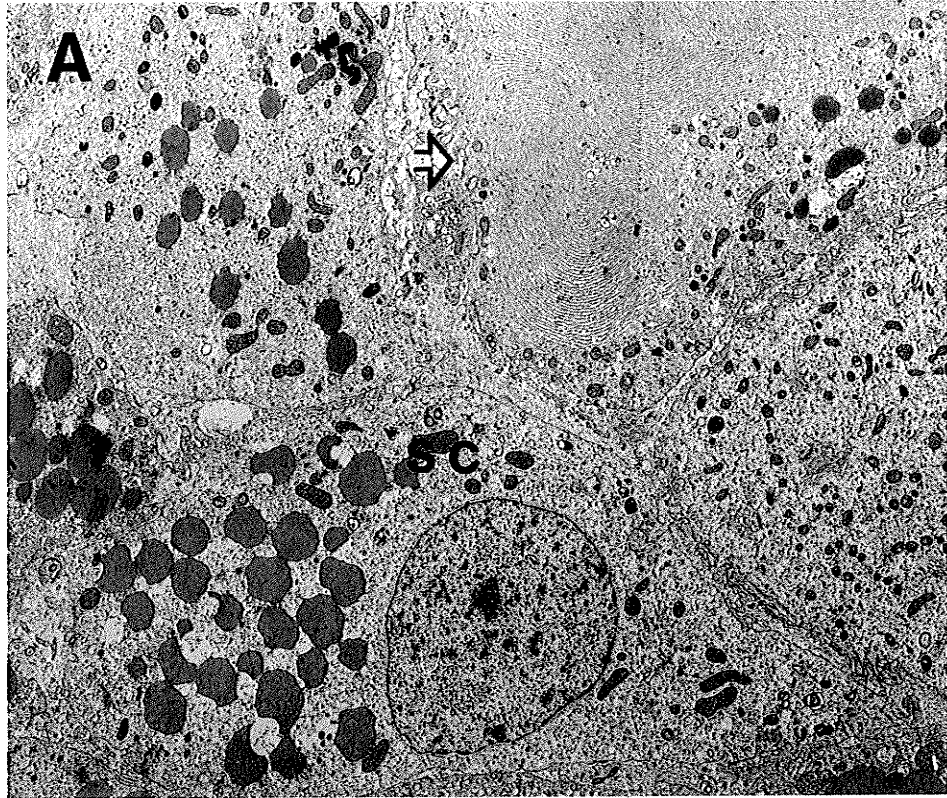
Figure 6. Transmission electron microscopy of porcine large
luteal cells during the estrous cycle.

A. Large and small cells from a corpus luteum of day 15
    during the estrous cycle. Large cells still contained
    numbers of small mitochondria, lipid droplets, but
    mitochondria, lipid droplets and SER had almost
    disappeared in small cells. Arrows indicate small cells
    and others are large cells. X3000.

B. Cells from a corpus luteum of day 18 during the estrous
    cycle. Very few intact cells were apparent. X3300.
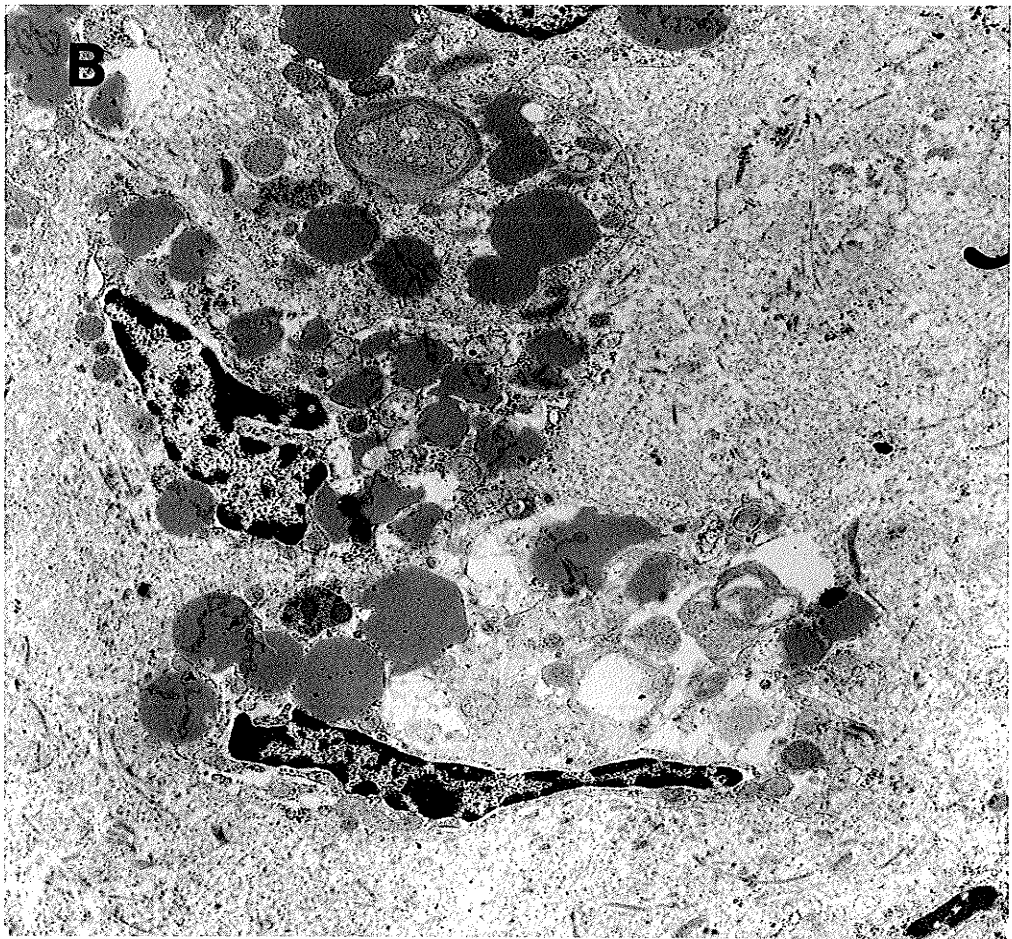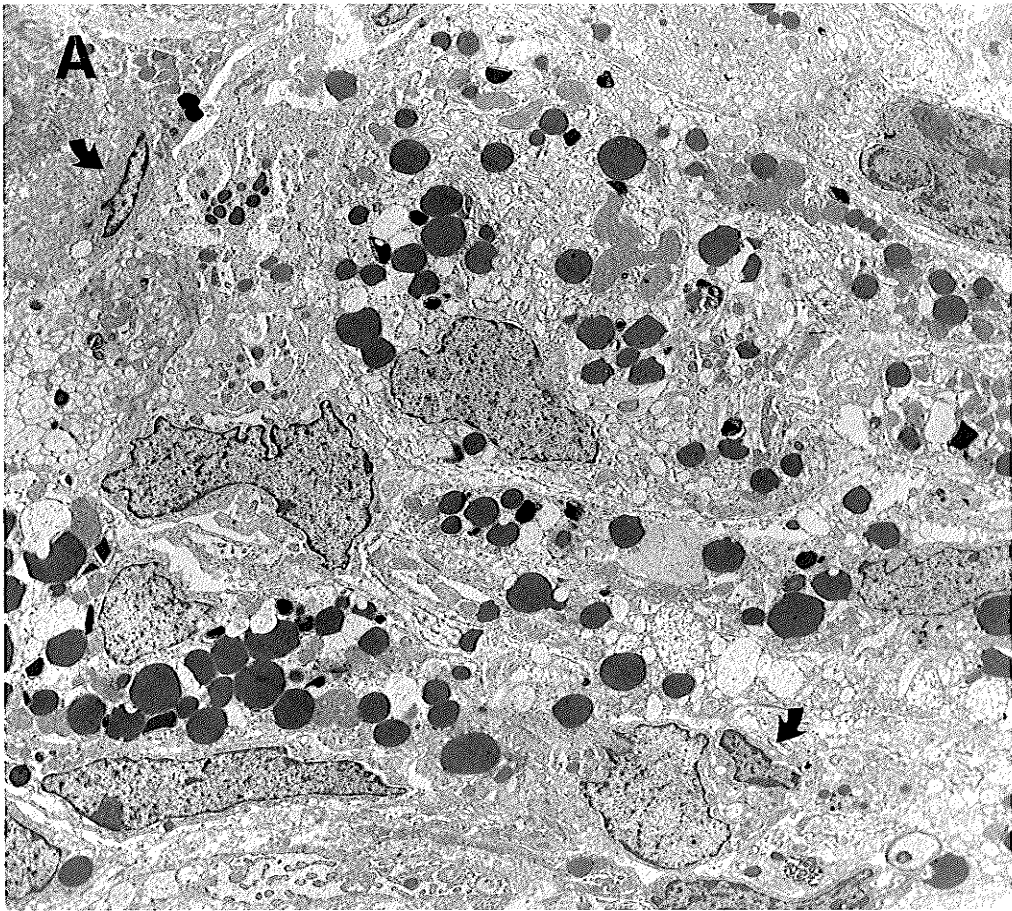
MANUSCRIPT II

Protein Kinase C Activity and Its Effect on Progesterone Production in Large and Small Porcine Luteal Cells

Abstract: Protein kinase C (PKC) activity and its effect on P4 production were investigated using the porcine large and small luteal cells on day 10 or 15 during the estrous cycle (day 0=onset of standing estrus). PMA significantly stimulated cytosolic PKC activity in both LC and SC. Twelve to sixteen hour incubation with 10 μM PMA resulted in cytosolic deficiency of PKC activity and a 57% (day 10) to 71% (day 15) translocation of PKC activity to membranes in LC. In normal small cells there was less measurable total than cytosolic PKC activity. Progesterone production was stimulated by PMA from 60 (LC) to 150 (SC) min of incubation. However, by 12 and 24 h of incubation, P4 production was significantly lower than in control cells. Reincubation of PKC deficient cells with PMA did not influence P4 production in the LC but 0.1 μM PMA induced a moderate increase of P4 production by the SC. Of the natural activators tested, PS + Ca^{++} and PS + DG + Ca^{++} stimulated PKC activity. The results demonstrated that activation of PKC can increase short-term P4 production by the LC and SC from day 10 of the porcine estrous cycle. The decreased P4 at 12 and 24 h may result from cytosolic deficiency of PKC activity.

Introduction

The Ca^{++}-polyphosphoinositol protein kinase C (PKC) second messenger system has been implicated in the control of P4 production in ovarian tissue of various species (Hansel and Dowd, 1986). On the one hand, the evidence suggests that the PKC system plays a luteotropic role in bovine luteal cells (Hansel and Dowd, 1986; Brunsuig et al., 1986; Alila et al., 1888) and in human granulosa-lutein cells (Jalkaner et al., 1987). On the other hand, it has also been suggested that the PKC pathway is involved in luteolysis of ovine (Hoyer et al., 1988; Wiltbank et al., 1989; Hoyer and Marion, 1989; Wiltbank et al., 1990) and rat (Baum et al., 1987) luteal cells. The LH or $PGF_{2\alpha}$ was reported to increase inositol lipid turnover in bovine (Davis et al., 1987; Davis et al., 1987; Davis et al., 1988; Duncan and Davis, 1988), rat (Leung et al., 1986; Lahav et al., 1988], ovine (McCann and Flint, 187) and human (Davis et al., 1989) luteal tissues. The products of inositol lipid turnover, DG and IP3, are involved in activation of PKC (Bell, 1986). However, the relationship between PKC systems and steroidogenesis in porcine luteal tissue has yet to be elucidated.

Porcine luteal tissue is unique in its resistance to $PGF_{2\alpha}$ in vivo over much of the cycle (Connor et al., 1976; Britt, 1987) and low responsiveness of its luteal cells (both LC and SC) to LH before or on day 10 (Hunter, 1981; Buhr, 1987; Agu, 1990; MSI). PKC activity has been found in luteal tissue from

several species (Davis and Clark, 1983; Clark et al., 1983; Wiltbank et al., 1989) including pigs (Noland and Dimino, 1986; Wheeler et al., 1987), but any differences related to cell type in porcine luteal tissue have not been defined. Furthermore, as far as we know, the effect of PKC activation on P4 production by porcine luteal cells has only been reported from our laboratory (Yuan and Connor, 1991).

The present experiments were designed to determine: 1) the effects of PMA on P4 production by large and small porcine luteal cells at mid-cycle (day 10) when they are considered to be relatively insensitive to luteotropic and luteolytic compounds and 2) the total and cytosolic PKC activity in large and small porcine luteal cells at mid-cycle (day 10) and at the late luteal phase (day 15) when the corpus luteum is more susceptible to luteolysins such as $PGF_{2\alpha}$.

Materials and Methods

Preparation of cells. Cell preparation was basically as described in MSI. In brief, corpora lutea were collected from cyclic gilts on day 10 or 15 of the estrous cycle (first day of standing estrus = day 0) and placed immediately in chilled fresh, sterile media comprised of Ham's F-12 Nutrient Mixture (10.6g/1L, GIBCO, Grand Island, New York) containing 1.176 g of $NaHCO_3$, .014 g polymyxin, .1 g streptomycin, .002 g insulin (Sigma, St. Louis), .00004 g hydrocortisone, .005 g transferrin (Sigma, St. Louis) and .0025 g metronidazole

(Sigma, St. Louis) per litre of deionized water. Aseptic techniques were employed and samples were kept on ice or at 4°C unless stated otherwise. The CL were decapsulated, weighed, chopped finely, and placed in 5 ml of media / g of tissue. Dissociation was carried out using sequential incubation (2 X 45 min; 1 X 30 min) in media containing 2 mg/ml collagenase (Type V, Sigma, St. Louis) in a shaking water bath at 37°C. Pellets formed from centrifugation of the retained supernatant were resuspended in the incubation media containing EGTA (1 mM, Sigma, St. Louis) and incubated at 37°C for a further 10 min. Final washing of the resulting cells with media was followed by layering cells onto a discontinuous Ficoll gradient (Pharmacia LKB) at room temperature. After 45 min, Ficoll layers with cells were filtered, pelleted (5 min., 500 g) and washed four times with fresh media. The cells were counted, viability checked using trypan blue exclusion and resuspended in the media with 10% heat deactivated bovine calf serum (Sigma, St. Louis) and treatment compounds as identified for each experiment. Total volume per well was 1 ml.

Experiment 1. Time series study (Figure 1). This was set up as a completely randomized block to look at the influence of PMA on progesterone production by normal cells over time. Cells from day 10 (n = 4 pigs) were resuspended at the rate of 3 X 10^4 large cells or 2 X 10^5 small cells / ml of incubation media. The cells were plated (24 well plate-Becton Dickinson Co., Lincoln Park, New Jersey) and incubated with or without

PMA (0, 1 and 10 μM). The media in culture plates were collected at 20 minute intervals during the first 2 h, at 30 minutes for 1 h, and then at 12 h and 24 h. The media were stored at -20°C until P4 was measured by RIA. All levels of PMA were tested in triplicate for luteal cells from each pig.

Experiment 2. The study of P4 in PKC deficient cells (Figure 1). Since 12-16 h incubation with PMA resulted in PKC deficient cells from ovine CL (Wiltbank et al., 1989), this study was designed to investigate whether the PKC deficient cells could still respond to PMA with P4 production. Cells were resuspended at the same rate as in Experiment 1, incubated with or without PMA (10 μM) for 12 to 16 h, and then washed two times with fresh media. The cells were then reincubated with or without PMA (0, 0.01, 0.1, 1 μM) for 2 h. After 2 h incubation, the media from the culture plates were collected and stored at -20^{0}C for P4 RIA.

Experiment 3. Measurement of PKC activity. In order to understand the characteristics of PKC in different size cells and the relationship between PKC and P4 production, PKC activity was stimulated and determined using natural and pharmacologic PKC activators. Corpora lutea from the ovaries of two gilts were used in each assay on days 10 (n = 8 pigs) and 15 (n = 8 pigs) of the estrous cycle. The assay was repeated four times for each day (day 10 or 15). The cells were resuspended in separate fractions at the rate of 2 X 10^6 LC or 2 X 10^6 SC / ml of incubation media with 10% heat

deactivated calf serum, placed in 28.6 X 104 mm sterile polycarbonate tubes (Nalge Company, Rochester) and incubated at 37°C (95% air/5% CO_2/100% humidity) for 12 to 16 h (overnight) with or without 10 μM PMA (Sigma, St. Louis) and /or H-7 (Sigma, St. Louis). Following overnight incubation, cells were washed twice with the media and either used directly for the PKC assay or incubated a further 2 h with or without 10 μM PMA and /or H-7.

PKC activity was determined using Amersham's PKC assay system (Figure 2, Amersham, Code RPN 77). This assay method is similar to other methods of PKC measurement (Noland and Dimino, 1986; Wheeler and Veldhuis, 1987; Wiltbank et al., 1989) except for the substrate, a peptide (Amersham) with greater specificity for PKC and less for cAMP dependent protein kinase than the histone IIIS commonly used (Noland and Dimino, 1986; Wheeler and Veldhuis, 1987; Wiltbank et al., 1989). This enzyme assay is based on the PKC catalytic transfer of the γ-phosphate group of [^{32}P]-ATP to the peptide. In preparation for the assay, the cells were centrifuged (500xg), washed twice using fresh media, and then transferred to numbered polystyrene tubes (12 X 75 mm) with 2 ml ice-cold buffer. The buffer (pH 7.5) contained 20 mM tris(hydroxymethyl)aminomethane (Sigma, St. Louis), 5 mM EDTA, 10 nM EGTA, 10 nM benzaridine, 0.1% w/v ß-mercaptoethanol, 50 μg/mol PMSF (Sigma, St. Louis) and 100 μl leupeptine (Sigma, St. Louis). Cells in the buffer were sonicated for 15 seconds

(Sonic Diemembrator, Model 300, Fisher). For cytosolic PKC activity, the cell lysate was centrifuged at 16,000xg for 15 minutes at 4°C in a microcentrifuge (EC CENTRA-M, International Equipment Company, USA). The PKC in the supernatant obtained following centrifugation was termed the cytosolic PKC (Figure 3). For total PKC activity (Figure 3), the cell lysate was first incubated with the buffer containing 0.2% Triton X-100 (Sigma) for 45 minutes to solubilize the membrane-bound PKC (Wheeler and Veldhuis, 1987; Wiltbank et al., 1989), and then centrifuged at 16,000xg for 15 minutes at 4°C in the microcentrifuge. The PKC activity in this supernatant was termed total PKC activity.

All assay components were brought to 25°C immediately prior to beginning the assay. The reaction mixture (total 75 μl) included 25 μl supernatant, 25 μl magnesium [γ-^{32}P]-ATP (2 mCi/ml, Amersham, Code PB 168) and 25 μl component mixture (Amersham RPN 77). The component mixture consisted of 900 μM peptide, 30 mM dithiothreitol (DTT), 12 mM Ca^{++}, 45mM magnesium/150 μM ATP. The mixture also contained either (a) no extra compounds (basal PKC activity) or (b) putative activators of PKC: 10 μM PMA, 10 μM DG and 10 μM PS or (c) the inhibitor H-7 (10 μM) or (d) 4α-phorbol ester (10 μM, Sigma, St. Louis) or dbcAMP (dibutyryl cAMP, 10 μM, Sigma, St. Louis). The reaction was carried out at 25°C in a water bath for 15 minutes and terminated by the addition of 100 μl stop reagent (a diluted acid, Amersham RPN 77). Following

termination of the reaction, 125 μl aliquots were removed and placed on numbered binding papers. The papers were individually washed using 5% v/v acetic acid and then placed into individual scintillation vials and counted using a liquid scintillation counter (Wallac Oy, Finland). All test levels were performed in triplicate. Non-specific binding and basal PKC activity were checked in each assay.

Measurement of P4. The concentration of P4 was determined by RIA (Yuthasastrakosol et al., 1974) without ether extraction using P4 antisera raised in rabbits (A18, N. Rawlings, U. of Saskatchewan). The inter- and intra-assay coefficients of variation were 9% and 6% respectively. Progesterone was expressed as ng P4.2X10^5 (SC) or 3X10^4 (LC) cells^{-1}. The mean sensitivity of the assay at 95% binding was 12 pg/tube.

Statistical Analysis. For experiment 1, 2 and 3, the compounds were used in a completely randomized block design. Blocks consisted of individual pigs on days 10 or 15. The error term used to test treatment effects was the treatment by block interaction term. Data were analyzed by the General Linear Models Procedure (SAS). Treatment effects were evaluated for each cell type on each day. Differences were tested using the Bonferroni test (SAS, 1985). Values were presented as LSM ± sem.

Results

Cell viability in the three experiments, as determined by 0.4% trypan blue exclusion, was between 80% and 85% after isolation. After overnight incubation with or without PMA, cell viability was between 71% and 82%. PMA treatment did not influence cell viability. The contamination of the SC by the LC was 0.27±0.11% (x±sem) and the contamination of the LC by the SC was 18±1.84% (x±sem).

Experiment 1 Time series study. The frequent samples of media collected during the first 3 h showed that both 1 and 10 μM PMA increased P4 production by the LC within 180 min. The P4 levels secreted by the LC remained significantly greater than controls between 60 and 150 min (1 μM PMA), and at 120 min (10 μM PMA) of incubation (P < 0.05, Figure 1 A). However, by 12 and 24 h, both 1 and 10 μM PMA had decreased P4 production by the LC (P < 0.05, Figure 2 A). In the SC, 1 μM but not 10 μM PMA increased P4 production by 150 min (P < 0.05, Figure 1 B). At 12 and 24 h, 10 μM PMA decreased P4 production by the SC (P<0.05, Figure 2 B).

Experiment 2. The cells incubated with 10 μM PMA for 12-16 h were termed PKC deficient cells patterned after the PKC deficient ovine cells of Wiltbank et al. (1989). Production of P4 was similar in normal and preincubated LC but was lower in preincubated SC than their normal control cells. In PKC deficient cells reincubation with 0.1 μM of PMA for 2 h stimulated P4 production (P < 0.05) in the SC but not in the

LC (Figure 3 A and B). Only PMA at 1 μM increased P4 production (P < 0.05) by the normal (control) LC and SC (Figure 3 A and B).

Experiment 3. Protein kinase C activity. Total PKC activity (cytosolic + membrane-bound) in the LC was similar on days 10 and 15 but exceeded that of small cells by 18 (day 10) and 32 (day 15) fold (Figures 4 and 5). Cytosolic PKC activity in the LC was 62% and 71% of total PKC activity (Figure 4 A and B) on days 10 and 15 respectively. Incubation with PMA for 12 to 16 h resulted in a significant translocation of 57% (day 10) and 72% (day 15) of cytosolic PKC activity to membranes in the LC (P < 0.05, Figure 4 A and B) compared to control cells. The PKC activity remaining after the 12 to 16 h preincubation was similar to basal PKC activity measurable when no PKC activators were included in the assay (Table 1). In the SC (Figure 5 A and B), the cytosolic PKC activity exceeded measurable total PKC activity by 71% (d 10) and 139% (day 15). Incubation with PMA decreased cytosolic PKC activity by 83% (day 10) and 79% (day 15) from corresponding control SC (Figure 5 A and B) to basal levels. Total PKC was similar in control and preincubated cells.

The effects of incubation for 15 min with PKC activators and cofactors on cytosolic PKC activity are shown in Tables 1 and 2. Basal (nonstimulated) cytosolic PKC activity was low in both LC and SC on days 10 and 15. PMA strongly stimulated cytosolic PKC activity (P < 0.05) above basal levels in both

LC and SC from days 10 and 15. Of the other compounds tested, PKC activity in cells was not influenced by 4α-phorbol, dbcAMP, Ca^{++} or DG + Ca^{++} on day 10 (Table 1). Only PS + Ca^{++} and PS + DG + Ca^{++} had a modest stimulatory effect. On day 15, PKC activity was modestly stimulated over basal activity by 4α-phorbol, PS + Ca^{++} and PS + DG + Ca^{++} (Table 1).

Use of the PKC inhibitor H-7 inhibited immediate PMA-stimulated cytosolic PKC activity ($P < 0.05$) in the LC and SC from days 10 and 15 (Table 2). However, two hour incubation of cells with H-7 did not block PKC activity. Two hour incubation of cells with PMA with or without H-7 produced a significant decline in measurable cytosolic PKC activity (Table 2, $P < 0.05$) similar to the effect of PMA alone.

Discussion

In pursuit of possible mechanisms regulating porcine luteal function, we investigated the influence of PKC activation in separate fractions of large and small luteal cells during the mid-luteal phase and the relative localization of PKC activity (total vs cytosolic) in cells from mid-cycle and those from the late luteal phase. PMA, a well documented activator of PKC in the corpus luteum (Davis and Clark, 1983; Noland and Dimino, 1986; Wheeler and Veldhuis, 1987; Wiltbank et al., 1989), was used initially. In the present study, both cell types responded to low (1μM) and LC to high (10μM) levels of PMA by increasing P4

production during short-term incubation. The LC appeared more sensitive to PMA stimulation both in the short and long term incubation but PKC deficient SC retained enough responsiveness to be modestly stimulated by .1 μM PMA.

Results of PKC activation on P4 production in luteal cells from other species are paradoxical and seem to depend on such factors as species, cell type, incubation time and whether or not luteotropic agents are present (Alila et al., 1988; Wiltbank et al., 1989; Benhaim et al., 1987; Benhaim et al., 1990). A number of hormones depend upon the PKC system for their intracellular actions (Nishizuka, 1986). However, the mid luteal phase porcine corpus luteum is considered to require little if any luteotropic support (Hunter, 1981; Buhr, 1987; Agu, 1990; Yuan and Connor, 1990). Both the large and small porcine luteal cells from mid-cycle demonstrated minimal responsiveness to LH (Yuan et al., 1992), although porcine mixed luteal cell cultures have shown luteotropic responses to LH (Wiesak and Foxcroft, 1992). The LH, however, is generally considered to affect P4 production through the cAMP and protein kinase A pathways (Niswender and Nett, 1988; Hansel et al., 1991). What then could be a hormonal link between increased PKC activation and corresponding P4 production in the porcine corpus luteum? One possibility is $PGF_{2\alpha}$. The $PGF_{2\alpha}$ tended to affect a transient increase in P4 production in the porcine large cells from day 10 (MSI) and from the bovine small cells (Alila et al., 1988; Benhaim et al., 1987;

Alila et al., 1988), and to enhance both basal and LH stimulated P4 production in mixed, dispersed porcine luteal cells (Mattioli et al., 1985). It has been demonstrated that AA, the precursor of $PGF_{2\alpha}$, stimulated P4 production by bovine LC on day 8 of the estrous cycle (Lafrance and Hansel, 1992). AA has been reported to stimulate oxytocin production by bovine LC and P4 production by both LC and SC on day 8 of the estrous cycle. Inhibition of the lipoxygenase metabolic pathway by nordihydroguaianetic acid abolished the AA-induced release of both oxytocin and P4 production in the bovine CL (Lafrance and Hansel, 1992). Thus, $PGF_{2\alpha}$ may be one hormone which can exert a luteotropic effect through the PKC second messenger system in the porcine luteal cells on day 10 during the estrous cycle. Whether or not this response is physiologically significant remains to be defined. But it may be one explanation for the porcine corpus luteum being insensitive to the luteolytic effects of $PGF_{2\alpha}$ until after day 12 of the estrous cycle (Britt, 1987). Three distinct isoforms of PKC were identified in pig luteal cytosol (Wheeler and Veldhuis, 1989), which may be readily activated producing distinct actions (Kosaka et al., 1988) which culminate in luteolysis. In fact, activation of PKC in day 15 porcine luteal cells resulted in a dramatic drop in P4 production (Yuan and Connor, 1992a) within 2 h of incubation.

Longer incubation of cells from day 10 with PMA resulted in depressed P4 production at 12 and 24 h. This could be a

true inhibition, the result of chronic exposure to PMA (Hansel et al., 1991), 'down regulation' or PKC deficiency (Wiltbank et al., 1989). Preincubation of cells with PMA for 12-16 h left the LC unresponsive to reincubation with PMA and the SC only moderately responsive to low levels of PMA. Measurement of PKC activity demonstrated that prolonged exposure of the day 10 or day 15 luteal cells resulted in relative cytosolic deficiency of PKC activity as reported in other species (Wiltbank et al., 1989). Therefore, it would appear that the predominant short-term effect of activation of the PKC system in day 10 porcine luteal cells is to increase P4 production.

In the present study PMA at 10 uM resulted in more depressed P4 production than did PMA at 1 uM at 12 to 24 h of incubation with day 10 luteal cells. PMA may inhibit some steroidogenic enzymes for P4 production (Wiltbank et al., 1989). More recently, PKC has been implicated as a link between intracellular signal transduction pathways. This "cross talk" provides a means of communication between distinct signalling systems and the capability to regulate responses based on past and present signals (Mouslay, 1990). Most often, PKC activation results in attenuation in cell responsiveness, either through homologous receptor desensitization or through modification of an unrelated signalling system (Mously, 1990). However, instances of enhancement or stimulation of new actions have been reported. In rat submandibular cells, PKC can desensitize adenylate

cyclase by direct phosphorylation of the ß receptor, but potentiate the cAMP response by a post-receptor mechanism (Fleming et al., 1992). The "cross talk" effect has not been reported in the luteal tissues but it may be present when PKC is activated in the luteal cells. In long term incubation of the present study, PKC may phosphorylate $PGF_{2\alpha}$ receptor and desensitize phospholipase C through its different isozymes (Nishizuka et al., 1991), and result in less DG and IP3 production. This effect could be greater with higher levels of PMA (10 μM) and result in more depressed P4 production. Another possible way is that PKC may desensitize LDL receptor and stop LDL transportation to the cell in long term incubation. The long term inhibitory effect of PMA on P4 production on day 10 should be investigated further.

Like the noted differences in responsiveness of LC and SC and to some hormones (Alila et al., 1988; Wiltbank et al., 1990; Benhain et al., 1990; Wiltbank et al., 1991), the evaluation of PKC in LC and SC revealed some distinct cell type difference in PKC activity. Total PKC activity was 18 and 32 fold greater in the LC than the SC on days 10 and 15 of the estrous cycle, respectively. Total PKC activity remained similar in the LC on days 10 and 15, but in the SC day 15 PKC activity was 55% that of day 10. The SC were unique in that the measurable cytosolic PKC was greater than total measurable PKC activity. This would imply that in the SC as in some other cells (Thomas et al., 1987), a large portion of PKC is

associated with the intracellular membrane fraction of organelles such as mitochondria or endoplasmic reticulum and is more sensitive to destruction by the Triton X 100 treatment required to solubilize membrane-bound PKC. The significance of these cell type differences is not clear. However, the large and small cells do respond differently, especially on day 15 (MSI) when we have noted the SC more advanced in luteolysis and more responsive than the LC to luteolytic effects of $PGF_{2\alpha}$ and oxytocin (MSI).

Preincubation of the LC and SC with PMA resulted in cytosolic PKC deficiency and decreased responsiveness of preincubated cells to further PMA challenge. The translocation of PKC to the membrane fraction is considered to be evidence of PKC activation (Bell, 1988; Witters and Blachshear, 1987). We did not observe a decline in total PKC activity with prolonged PMA treatment as seen in ovine luteal cells and other cells (Wiltbank et al., 1989; Thomas et al., 1987). However, this proteolytic degradation does not occur in all cell types (Cooper et al., 1982). Our results demonstrate that PMA stimulation of PKC activity in the porcine large cells, at least, involves translocation and binding of PKC to the plasma membrane where it remains, leaving the cell functionally PKC deficient. Thus, our observation of decreased progesterone production after 12 and 24 h incubation with PMA may result from a PKC deficiency rather than prolonged PKC activation.

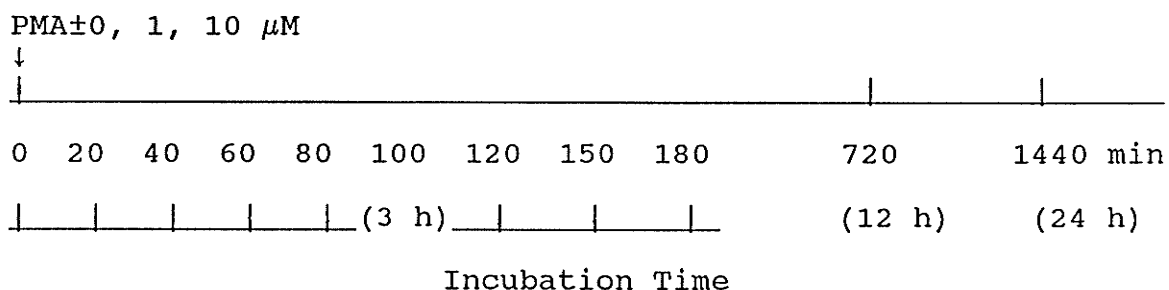Results with natural activators and cofactors of the PKC

system support the requirement of PKC for PS, DG and Ca^{++} for its activity. Likewise, as expected 4α-phorbol and dbcAMP had no significant influence on PKC activity on day 10 large and small cells. This indicates that the increased progesterone production on day 10 is largely through activation of the PKC rather than protein kinase A system. The increase in PKC activity seen when dbcAMP and, to a lesser extent, 4α-phorbol were used in the incubation on day 15 may represent the emergence of another PKC isozyme. This isozyme may then be associated with the different effect of PKC activation on progesterone production observed by day 15.

In summary, activation of PKC can increase short term progesterone production by the large and small luteal cells on day 10 of the porcine estrous cycle. The decreased progesterone at 12 and 24 h of incubation may be the result of prolonged PMA treatment resulting in the cytosolic deficiency of PKC activity. Conversely, the long term decline in P4 may have a normal physiological significance in need of definition. PKC activity was apparently associated with different cellular locations in small and large cells which may relate to the differential responsiveness of these two cell types to putative luteotropic and luteolytic agents. $PGF_{2\alpha}$ may be capable of stimulating P4 production through the

PKC system in the porcine luteal cells on day 10 during the estrous cycle, but this requires confirmation.

Figure 1. Incubation arrangements and times for studying the effect of PMA on progesterone production in the porcine large and small luteal cells on day 10 in experiments 1 and 2

Experiment 1

PMA±0, 1, 10 μM

0   20   40   60   80   100   120   150   180   720   1440 min

|___|___|___|___|___(3 h)___|___|___|___   (12 h)   (24 h)

Incubation Time

Experiment 2

a) Preincubated cells with PMA (PKC deficient cells)

PMA±10 μM
↓   Sample Wash   Sample

PMA±0, 0.01, 0.1, 1 μM

0 h   12 to 16 h   2 h

b) Normal Cells

No PMA
↓   Sample Wash   Sample

PMA±0, 0.01, 0.1, 1 μM

0 h   12 to 16 h   2 h
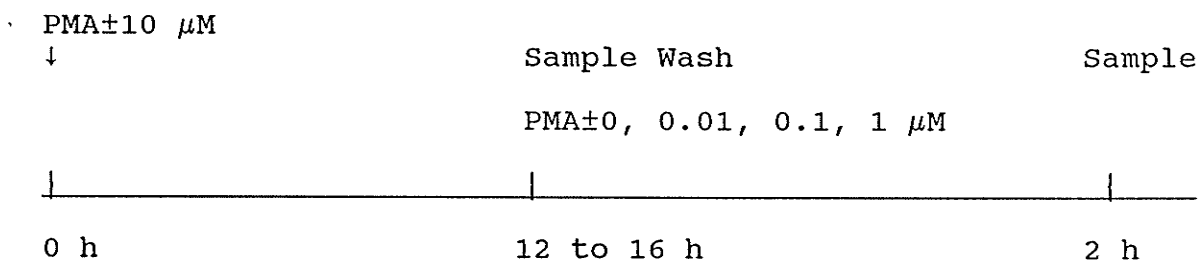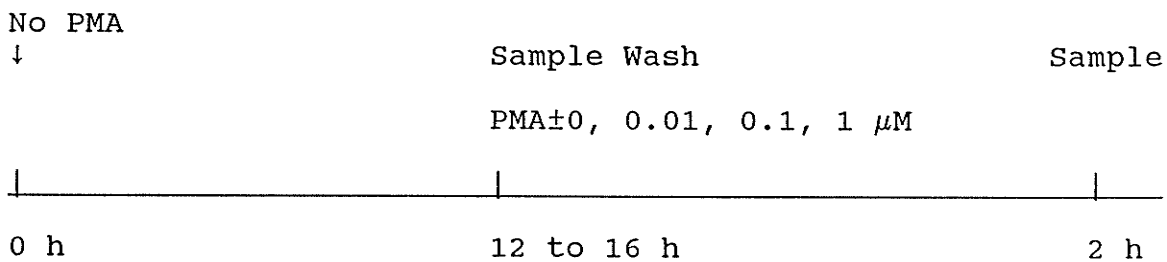
Incubation Time

Note : All test levels were done in triplicate

Figure 2. Protein Kinase C Assay Principle

(from Amersham Code RPN 77)

Figure 3. Cell incubation and treatment
for protein kinase C assay in experiment 3

Cells ± PMA (10 μM) for 12-16 H
↓

↓   ↓   ↓   ↓   ↓

Normal   Normal   Normal   PKC   PKC
                                 Deficient Deficient
Cells   Cells   Cells   Cells   Cells

↓

Reincubated
±H-7, PMA
or H-7+PMA
for 2 H

↓

Ultrasonicated for 15 min

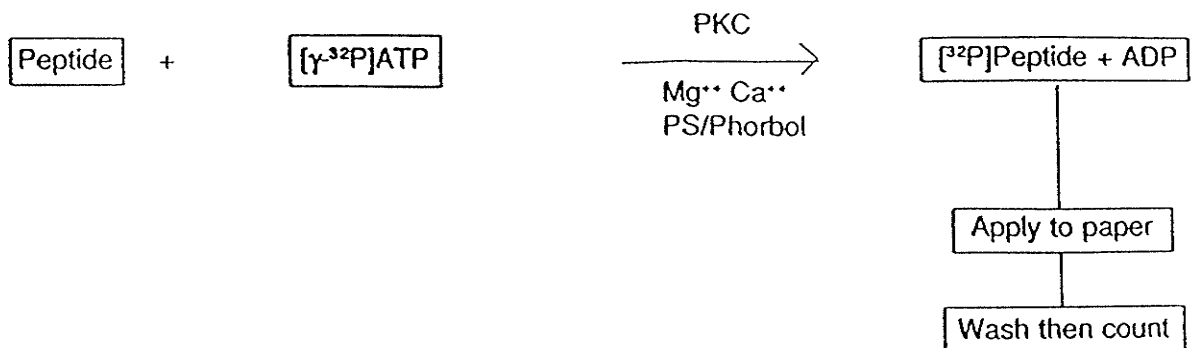↓   ↓   ↓   ↓   ↓

Centrifuged Centrifuged Triton   Centrifuged Triton
15,000xg   15,000xg   X-100   15,000xg   X-100
                          45 min   45 min

                          Centrifuged   Centrifuged
                          15,000xg   15,000xg

Cytosol   Cytosol   Cytosol   Cytosol   Cytosol
(Cytosolic (Cytosolic (Total   (Cytosolic (Total
PKC)   PKC)   PKC)   PKC)   PKC)

↓   ↓   ↓   ↓   ↓

PKC Assay PKC Assay PKC Assay PKC Assay PKC Assay

Figure 4. The effect of different doses of PMA (0, 1, 10 μM) on progesterone production (ng/3X10^{-4} large cells and ng/2X10^5 small cells, LSM ± sem) at 20 minute intervals within the first 3 h incubation in the large (A) and small (B) cells on day 10 during the estrous cycle (n = 4 pigs). * (P < 0.05) different from control level (0 μM PMA).
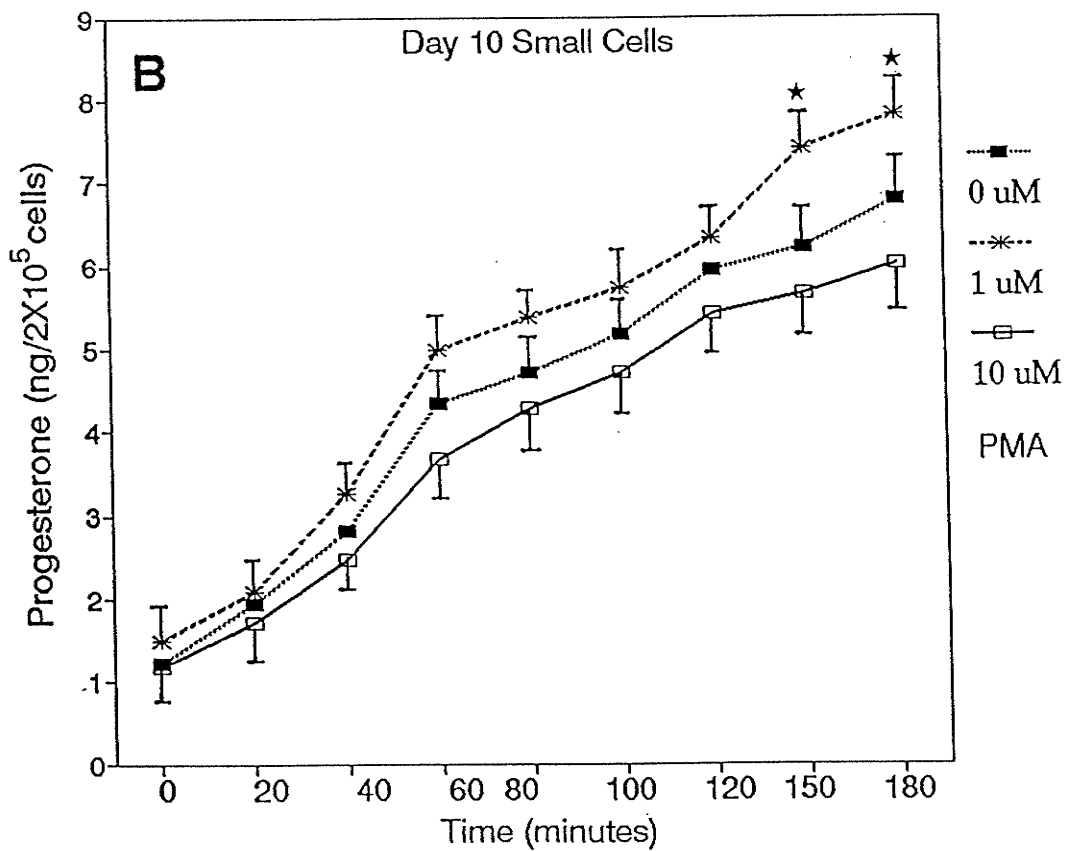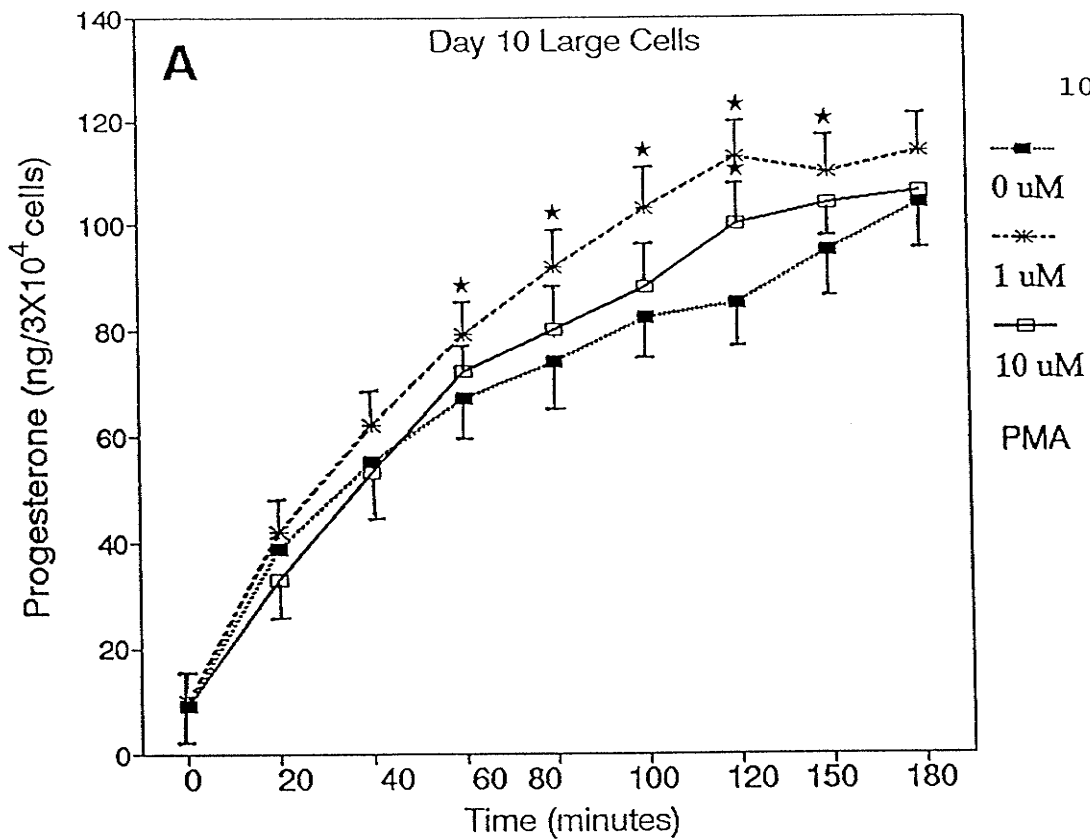
Figure 5. The effect of different doses of PMA (0, 1, 10 μM) on progesterone production (ng/3X10^4 large cells and ng/2X10^5 small cells, LSM ± sem) at 12 and 24 h in the large (A) and small (B) cells on day 10 during the estrous cycle (n = 4 pigs). * (P < 0.05) different from control level (0 μM PMA).
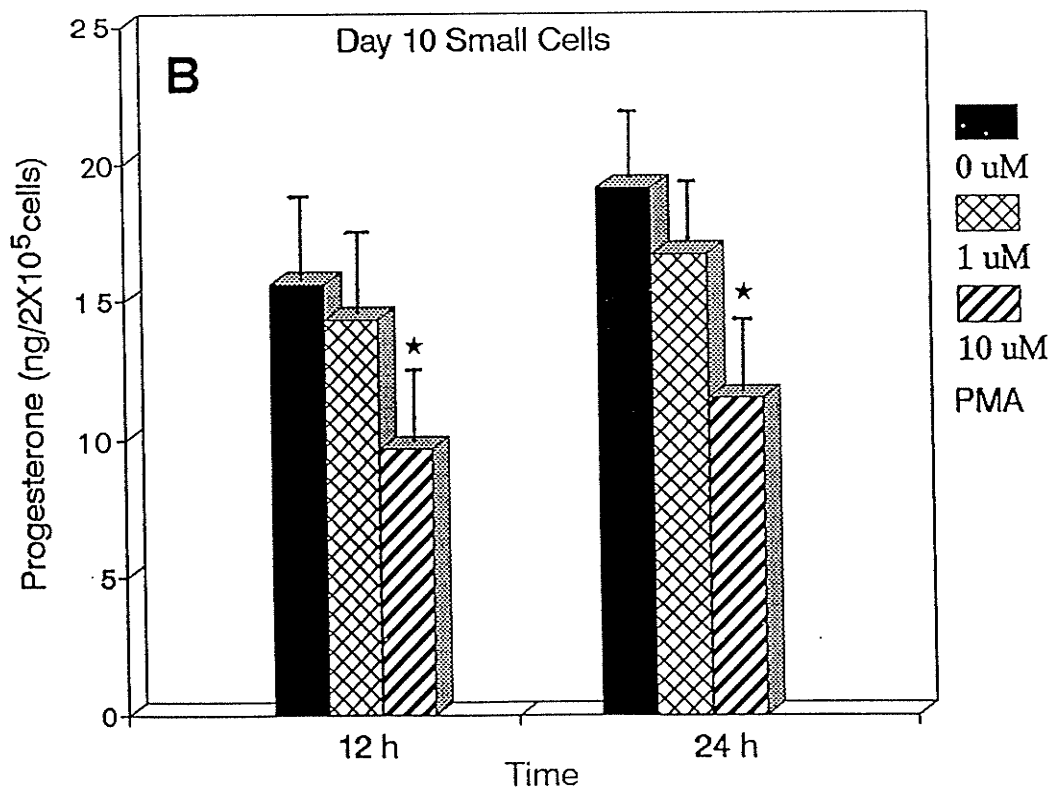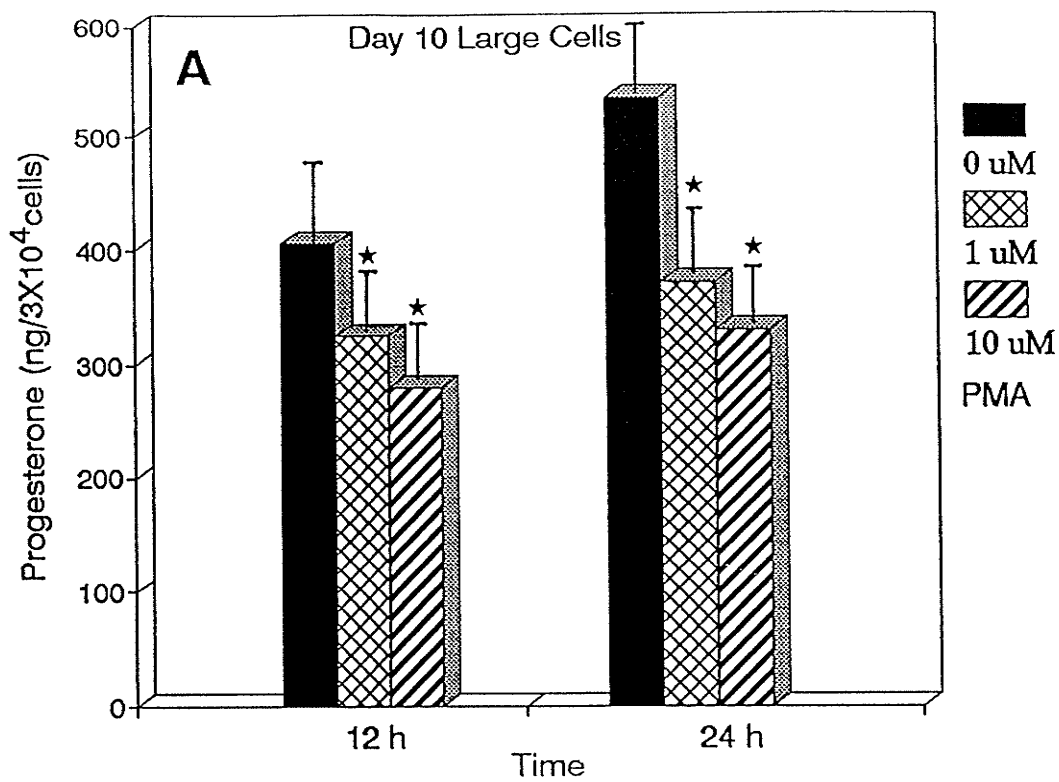
Figure 6. The effect of different doses of PMA (0, 1, 10) on progesterone production (ng/3X10^4 large cells and ng/2X10^5 small cells, LSM ± sem) in the normal and PKC-deficient large (A) and small (B) cells (n = 4 pigs). * (P<0.05) different from 0 μM level of PMA.

A

Day 10 Large Cells

104

B

Day 10 Small Cells

A Day 10 Large Cells 2 Hrs Incubation

Progesterone (ng/3X10^{4}cells)

Control   Preincubated with PMA

0 uM
0.01 uM
0.1 uM
1 uM
PMA

B Day 10 Small Cells 2 Hrs Incubation

Progesterone (ng/2X10^{5}cells)
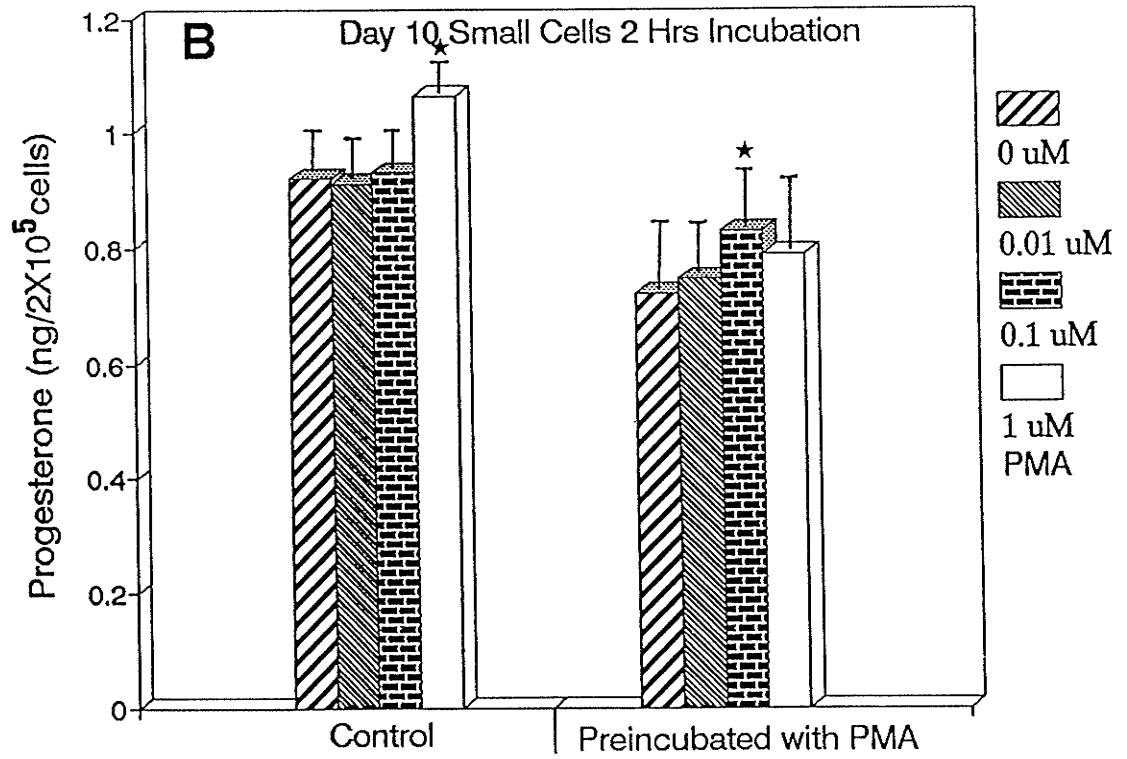
Control   Preincubated with PMA

0 uM
0.01 uM
0.1 uM
1 uM
PMA
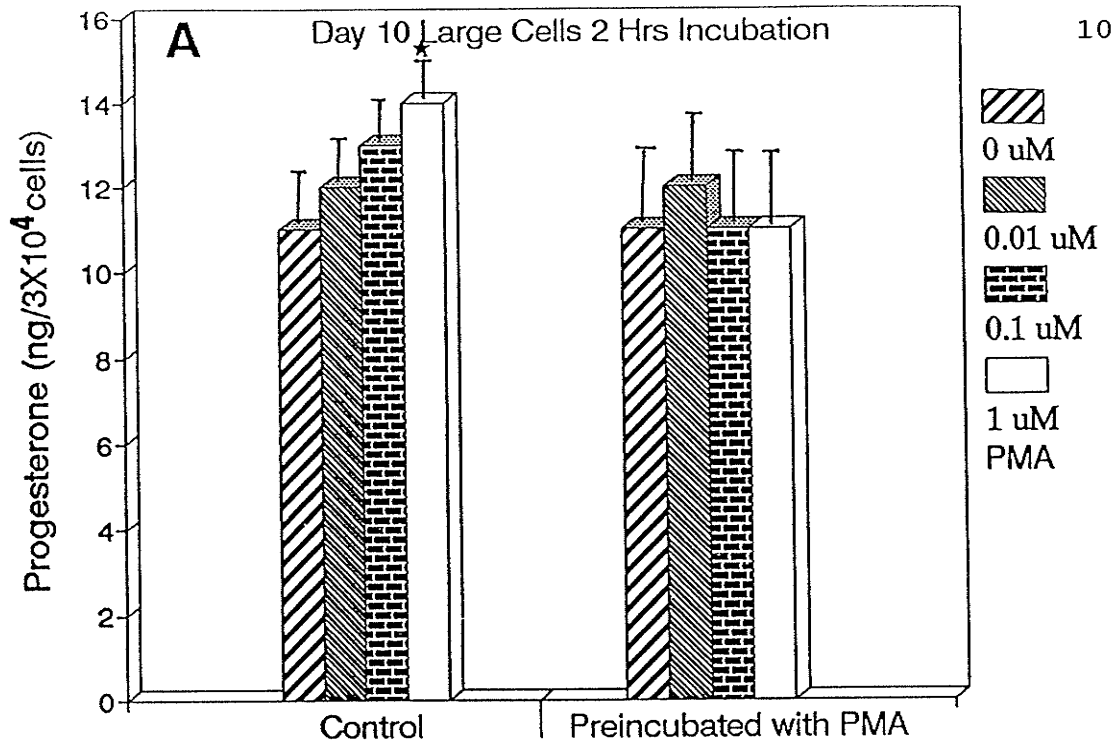
Figure 7. Influence of incubation with (preincubated) or without (control) 10 μM PMA for 12 to 16 h on the distribution of protein kinase C activity (pmole/min phosphorylated, LSM ± sem) in the large cells (2 X 10^6) on day 10 (A) and day 15 (B) (n = 8 pigs from each day). Columns that do not have a common letter differ significantly (P < 0.05).

Figure 8. Influence of incubation with 10 μM PMA for 12 to 16 h on the distribution of protein kinase C activity (pmole/min phosphorylated, LSM ± sem) in the small cells (2 X 10^6) on day 10 (A) and day 15 (B) (n = 8 pigs from each day). Columns that do not have a common letter differ significantly (P < 0.05).
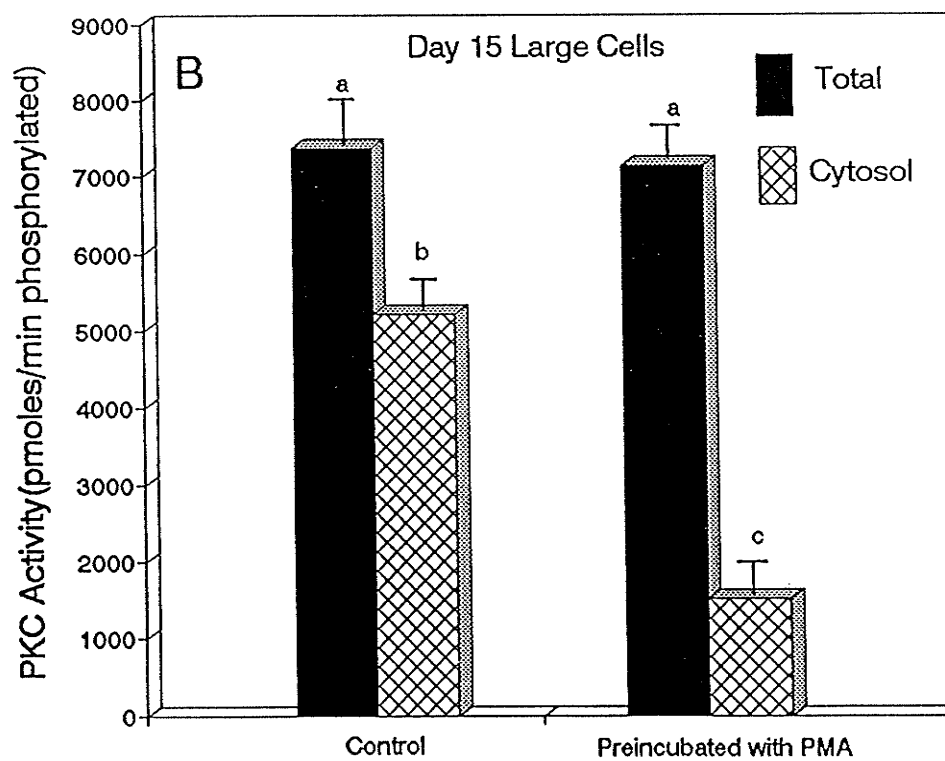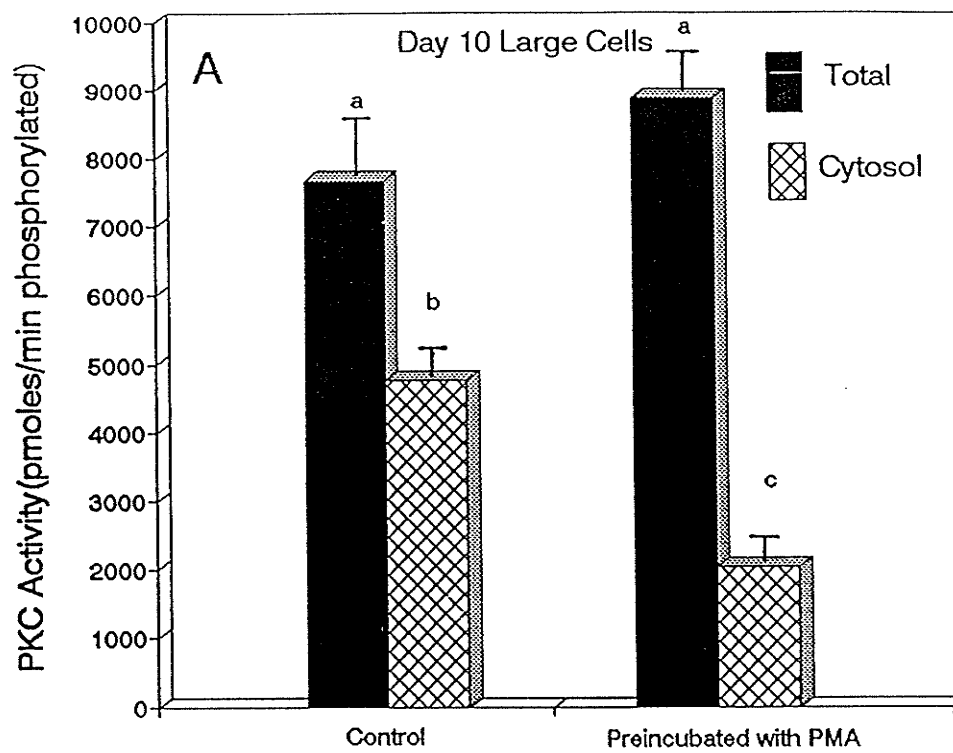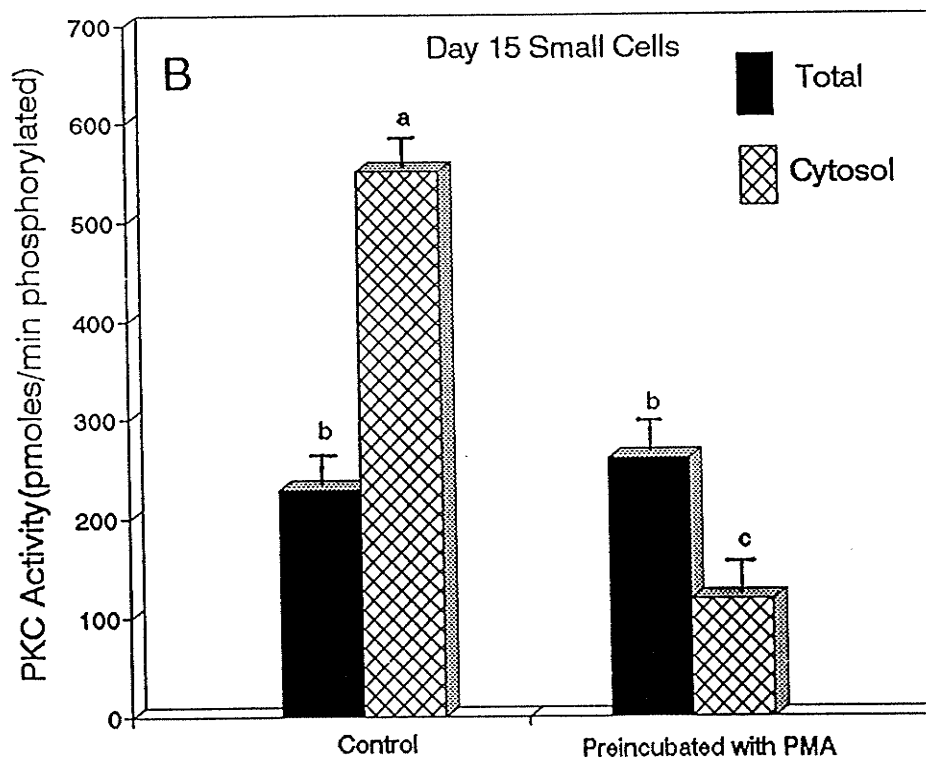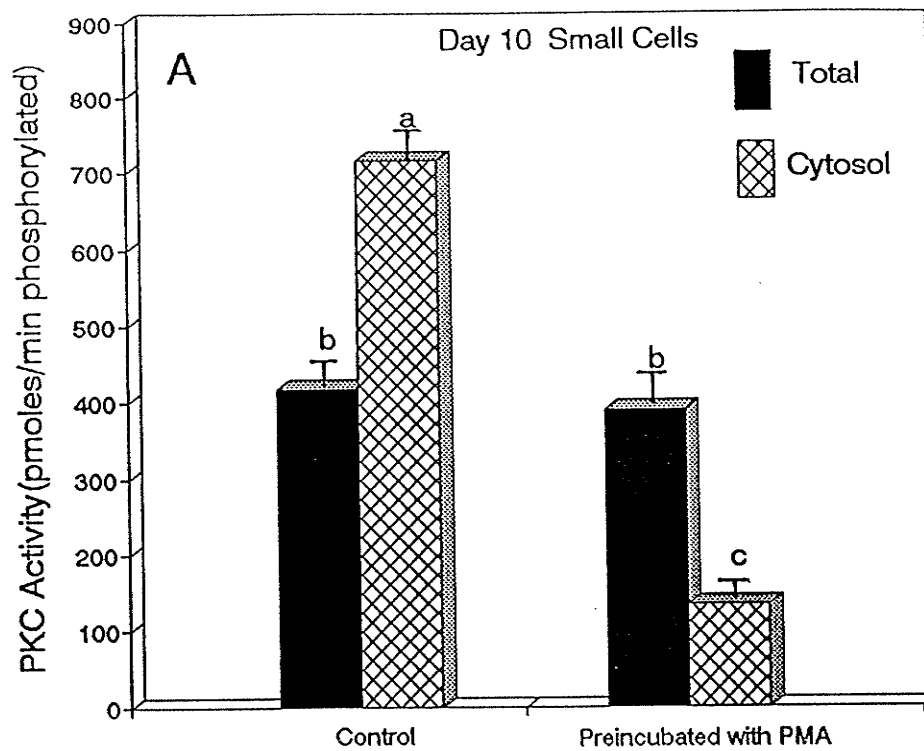
Table 1. Cytosolic protein kinase C activity stimulated by natural and pharmacologic PKC activators (pmole/min phosphorylated, LSM ± sem).

| | Large Cells ($2\text{X}10^6$ cells) | | Small Cells ($2\text{X}10^6$) cells) | |
	day 10	day 15	day 10	day 15
Basal	1433 ± 35^c	925 ± 206^d	133 ± 6^c	92 ± 8^d
PMA	4755 ± 119^a	5227 ± 205^a	716 ± 48^a	551 ± 38^a
4α-phorbol	1565 ± 66^c	1539 ± 139^c	139 ± 7^c	172 ± 37^c
dbcAMP	1562 ± 42^c	2803 ± 540^b	133 ± 6^c	309 ± 64^b
Ca^{++}	1432 ± 34^c	936 ± 207^d	127 ± 5^c	71 ± 10^d
$DG+Ca^{++}$	1508 ± 31^c	844 ± 146^d	133 ± 5^c	102 ± 14^d
$PS+Ca^{++}$	2131 ± 134^b	1409 ± 298^c	199 ± 20^b	197 ± 30^c
$PS+DG+Ca^{++}$	2115 ± 101^b	1562 ± 281^c	228 ± 22^b	205 ± 22^c

[a,b,c]Those not having a common letter within the columns differ ($P < 0.05$).

Table 2. The effect of incubation with or without H-7, PMA or PMA+H-7 for 2 h on cytosolic protein kinase C activity (pmole/min phosphorylated, LSM ± sem).

	Large Cells (2X10^6 cells)		Small Cells (2X10^6) cells	
	day 10	day 15	day 10	day 15
Control$^\$$	4758 ± 118^a	5633 ± 364^a	716 ± 41^a	550 ± 37^a
H-7$^\$$	3645 ± 54^b	3168 ± 316^b	430 ± 27^b	219 ± 46^b
H-7*	5322 ± 202^a	6099 ± 202^a	593 ± 17^a	571 ± 70^b
PMA*	1835 ± 25^c	1630 ± 44^c	161 ± 40^c	169 ± 22^c
PMA + H-7*	1257 ± 36^c	1615 ± 15^c	141 ± 13^c	143 ± 54^c

[a,b,c]Those not having a common letter within the columns differ (P < 0.05).

$^\$$The cells were incubated without any test compounds for 2 h, and then the cells were sonicated and the cytosol was used to test 10 μM H-7 effect on PMA-stimulated protein kinase C activity in protein kinase C assay system.

*The cells were incubated with 10 μM H-7, 10 μM PMA and PMA + H-7 (both of them were 10 μM) for 2 h, and then the cells were sonicated and the cytosolic protein kinase C activity was determined in protein kinase C assay system.

MANUSCRIPT III

Activation of Protein Kinase C has Distinctly Opposite

Effects on Steroidogenesis by Porcine Luteal Cells

at the Mid- and Late-luteal Phase

ABSTRACT: Large and small porcine luteal cells isolated from gilts on days 10 and 15 of estrous cycle were treated with PMA, 4α-phorbol ester and H-7 or PMA+H-7 for 2 (Day 15) or 3 (day 10), 12 and 24 h incubation. P4, testosterone and 17ß-estradiol were determined by RIA. The results showed that 10 μM PMA increased P4 production by the LC of day 10 within 180 min, but depressed P4 production by the 12 and 24 h incubation. In contrast, PMA significantly decreased P4 production at 2, 12 and 24 h incubation by both the LC and SC on day 15. H-7 blocked effects of PMA on days 10 and 15. 4α-phorbol ester did not have any effect on P4 production. PMA (10 μM) did not influence testosterone and 17ß-estradiol production by the LC and SC on days 10 and 15. The current results indicate for the first time that activation of PKC has a distinctly dual role on P4 production in the porcine luteal cells: stimulatory on day 10 but inhibitory on day 15. However, activation of PKC does not influence testosterone and 17ß-estradiol production. Similar dual effects on P4 production noted with $PGF_{2\alpha}$ (MSI) and activation of PKC may suggest that $PGF_{2\alpha}$ regulates P4 production through the PKC system.

Introduction

Isolated porcine luteal cells at mid cycle are not sensitive to the luteotropic influence of LH <u>in vitro</u> (Buhr, 1987; Agu, 1991; MSI) nor to the luteolytic effect of $PGF_{2\alpha}$ <u>in vivo</u> (Connor et al., 1976). From our first experiment (MSI), $PGF_{2\alpha}$ and oxytocin tended to increase P4 production in LC on day 10 but they decreased P4 production especially in SC on day 15. FSH also inhibited P4 production on day 15 (MSI). From the second group of experiments (Yuan and Connor, 1991; Yuan and Connor, 1992c), activation of PKC increased P4 production in porcine LC and SC on day 10 in short-term incubation.

There was no information available regarding the effect of PKC activation on steroidogenesis in porcine luteal cells from the late luteal phase (day 15) when they are more susceptible to luteolytic influences (MSI). Additionally, since PMA stimulation of porcine luteal cells on day 10 resulted in long term (12 and 24 h) decline in P4, one question was whether the decrease in P4 corresponded to an increase in other steroid hormones. Thus this experiment was designed to: 1) confirm the observation of the effects of PKC activation on P4 production by day 10 LC and SC; 2) determine whether PKC activation influenced production of testosterone and 17ß-estradiol; 3) extend these observation to day 15 luteal cells to determine the effects of PKC activation on steroidogenesis in the late luteal phase.

Materials and Methods

Preparation of cells. Cell preparation was basically as described in MSI and MSII. In brief, CL were collected from cyclic gilts on day 10 or 15 of the estrous cycle (first day of standing estrus = day 0) and placed immediately in sterile Hams F-12 Nutrient mixture (10.6g/1L, GIBCO, Grand Island, New York) containing 1.176 g of $NaHCO_3$, .014 g polymyxin, .1 g streptomycin, .002 g insulin (Sigma, St. Louis), .00004 g hydrocortisone, .005 g transferrin (Sigma, St. Louis) and .0025 g metronidazole (Sigma, ST. Louis) per litre of deionized water. Aseptic techniques were employed and samples were kept on ice or at 4°C unless stated otherwise. The CL were decapsulated, weighed, chopped finely and placed in 5 ml of media/g of tissue. Dissociation was carried out using sequential incubation (2 X 45 min; 1 X 30 min) in media containing 2 mg/ml collagenase (Type V, Sigma, St. Louis) in a shaking water bath at 37°C. Pellets formed from centrifugation of the retained supernatant were resuspended in the incubation media containing EGTA (1 mM, Sigma, St. Louis) and incubated at 37°C for a further 10 min. Final washing of the resulting cells with media was followed by layering cells onto a discontinuous Ficoll gradient (Pharmacia LKB) at room temperature. After 45 min, Ficoll layers with cells were filtered, pelleted (5 min., 500 g) and washed four times with fresh media. The cells were counted, viability checked using 0.4% trypan blue exclusion and resuspended in the media with

10% heat deactivated bovine calf serum (Sigma, St. Louis) and treatment compounds as identified for each experiment.

For P4, testosterone and 17ß-estradiol production (Figure 1), cells from days 10 (n = 6) or 15 (n = 4) were resuspended at the rate of $3X10^4$ LC or $2X10^5$ SC per ml of incubation media and placed in 24-well Multiwell Tissue Culture Plates (Bacton Dickinson & Company). Day 10 cells were incubated with or without 10 μM PMA (activator of PKC, Sigma), or H-7 (inhibitor of PKC, Sigma) or 4α-phorbol ester (a member of the phorbol ester family and nonactivator of PKC, Sigma). H-7 and H-8 are both derivatives of isoquinoline sulfonamide and they block protein phosphorylation by inhibiting ATP or GTP binding to protein kinase (Goodman et al., 1990). H-8 is at least twice as potent as H-7 with respect to PKA activation, while H-7 is at least twice as potent as H-8 at blocking PKC activation (Goodman et al., 1990). H-7 was therefore selected as a PKC inhibitor in the present study. It was found from MII that 10 μM PMA induced depressed P4 production in the SC so 10 μM was selected to treat day 10 luteal cells. The media from cultured cells of day 10 were collected at 30 min intervals during the first 3 h, and then at 12 h and 24 h. The cells from day 15 were incubated with 0, 1, 10 and 50 μM PMA, H-7, 4α-phorbol ester and H-7+PMA. The media from cultured cells were collected at 2, 12 and 24 h. The media were stored at -20^0C, and P4, testosterone and 17ß-estradiol were determined by RIA within two to three months. All test levels were done in

triplicate.

Measurement of P4, testosterone and 17ß-estradiol. The concentrations of P4, testosterone and 17ß-estradiol were determined by RIA using the method of Yuthasastrakosol et al., (1974) without ether extraction. The P4 antisera was raised in rabbits (A18, N,Rawlings, U. of Saskatchewan). Labelled P4 [1,2,3 H(N)] was purchased from Dupont (Boston). The inter- and intra-assay coefficients of variation for 14 assays were 11% and 8% respectively. P4 was expressed as ng P4·cells^{-1}·min^{-1} or (h). The mean sensitivity of the assay at 95% binding was 12 pg/tube.

For testosterone determination, the testosterone antiserum used had been raised in sheep (Sanford et al., 1974). Labelled testosterone [1,2,6,7-3H (N)] was purchased from Dupont (Boston). The inter- and intra-assay coefficients of variation for 6 assays were 15% and 9% respectively. Testosterone concentrations were expressed as ng testosterone·cells^{-1}·h^{-1}. The mean sensitivity of the assay at 95% binding was 12 pg/tube.

The 17ß-estradiol antiserum was raised in rabbits (NCR A18, N. Rawlings, U. of Saskatchewan). The [2,4,6,7,16,17-^{3}H (N)]-estradiol was purchased from Dupont (Boston). The inter- and intra-assay coefficients of variation for 3 assays were 13% and 8% respectively. The mean sensitivity of the assay at 95% binding was 6 pg/tube.

Statistical Analysis. The experiment was set up as a

randomized complete block design. Blocks consisted of individual pigs on each of days 10 and 15. The error term used to test treatment effects was the treatment by block interaction term. Data were analyzed by the GLM (SAS, 1985). Treatment effects of various compounds were evaluated for each cell type on each day. Differences were tested using the Bonferroni test (SAS, 1985). Values were presented as LSM ± sem.

Results

Cell viability, as determined by 0.4% trypan blue exclusion, was between 79% to 83% after isolation. After overnight incubation with or without PMA, cell viability was between 70% to 82%. PMA treatment did not influence cell viability. The contamination of the SC by the LC was 0.24±0.04% ($\bar{x}$±sem) and the contamination of the LC by the SC was 21±1.4% ($\bar{x}$±sem).

The samples collected at 30 min intervals showed that 10 μM PMA increased P4 production by LC of day 10 but not by SC between 120 and 180 min of incubation (P < 0.05, Figures 1 and 2). H-7 blocked the effect of PMA in H-7+PMA group (Figure 1). H-7 alone tended to decrease P4 production by the LC, but this was not statistically significant (P > 0.05). At 12 and 24 h incubation, depressed P4 production was found in the LC and SC incubated with PMA and H-7+PMA (P < 0.05, Figures 3 and 4). 4α-phorbol ester did not influence P4 production on day 10.

On day 15, PMA at 1, 10 and 50 μM decreased P4 production by LC at 2, 12 and 24 h incubation (P < 0.05, Figures 5,6,7). The SC P4 production decreased at 2 h by 1 and 10 μM PMA and at 12 and 24 h by 1, 10, 50 μM PMA (P < 0.05, Figures 5,6,7). For the most part, H-7 blocked the PMA effect in the H-7+PMA group at 2 h (Figure 5), but was not effective for the longer incubation periods (Figures 6 and 7). Exception to this were 1 μM PMA with LC and 10 μM PMA with SC at 24 h. 4α-phorbol did not influence P4 production in the LC and SC on day 15.

PMA and 4α-phorbol at 10 μM did not induce any change in testosterone production by the LC and SC at 2, 12 and 24 h incubation on days 10 or 15 (P > 0.05, Table 1). 17ß-estradiol concentration in PMA treated cells was so low that it was not measurable. Basal P4 concentration in the LC was approximately 40 times more than the testosterone level at 2 h incubation on day 10 of the estrous cycle. As well, basal P4 level in the SC was approximately 10 times more than testosterone level at 2 h incubation on day 10.

Discussion

Activation of PKC has been reported to increase P4 production by bovine SC from the middle of the cycle (Brunsuig et al., 1986; Benhaim et al., 1987; Alila et al., 1988; Benhaim et al., 1990) or by human granulosa-lutein cells (Jalkaner et al., 1987) but to inhibit P4 production by LC or LH-, forskolin-, dbcAMP-stimulated SC from the mid cycle of

sheep (Wiltbank et al., 1989; Hoyer and Marion, 1989). It appears that the different response of luteal cells to PKC activation depends on such factors as species, cell type, incubation time and presence or absence of luteotropic agents.

In our previous work, activation of PKC increased P4 production by the LC and SC from the porcine CL on day 10 in short-term incubation (MSII). The present study confirmed and extended these observations. Since the porcine LC contained more PKC activity than the SC (MSII) and were more sensitive to the stimulation of low and high levels (1 and 10 μM) of PMA (MSII and MSIII), it is therefore speculated that activation of PKC in the middle of the luteal phase predominantly acts on the LC to increase P4 production. Both porcine (MSII) and ovine (Wiltbank et al., 1989) LC contained more PKC activity and were more sensitive to PMA than SC. But activation of PKC had opposite effects, stimulatory in pigs (MSII and MSIII) and inhibitory in the ovine (Wiltbank et al., 1989). These species specific, cell type dependent effects of PKC on P4 production require further investigation.

Interestingly, PMA significantly inhibited P4 production by the LC and SC on day 15 at 2, 12 and 24 h incubation. The mechanism(s) responsible for this shift in PKC activity from being stimulatory on day 10 to being inhibitory on day 15 is not clear. One possibility may be the emergence of different PKC isozymes. It has been reported that different cell lines contain different PKC isozymes and these isozymes have

different roles depending on the cell lines (Gusovely and Gutkind, 1991). Seven isozymes in the PKC family have been found so far (Mischak et al., 1991). Three distinct isoforms of PKC in porcine luteal cytosol have been reported (Wheeler and Veldhuis, 1989). These isoforms may have distinct actions for initiating luteolysis.

From our previous work (MSI), LH had little effect on P4 production in isolated LC and SC populations during the estrous cycle. Similar results were reported in other studies (Buhr, 1987; Agu, 1991). However, $PGF_{2\alpha}$ and oxytocin tended to increase P4 production in LC on day 10 and to inhibit P4 production on day 15 especially in SC. $PGF_{2\alpha}$ initiates the breakdown of PIP2 to DG and IP3 in the bovine (Davis et al., 1987b; Davis et al., 1988), ovine (McCann and Flint, 1987), human (Davis et al., 1989) and rat (Leung et al., 1986; Lahav et al., 1988) luteal cells. DG and IP3 are the natural activators of PKC. On day 10, the porcine luteal cells, especially LC, responded to $PGF_{2\alpha}$, oxytocin and PMA. Thus, $PGF_{2\alpha}$ is capable of stimulating P4 production by the porcine luteal cells in the middle of luteal phase and its effect may be mediated through the PKC system.

On day 15, P4 production by the porcine luteal cells, especially SC, was inhibited by $PGF_{2\alpha}$ and oxytocin. Activation of PKC also inhibited P4 production in both LC and SC (MSIII). In situ, the LC may become more sensitive to $PGF_{2\alpha}$ and oxytocin by an important link between the LC and SC (MSI). This link

may involve a signal which is sent from the SC to the LC and is required for steroidogenesis and luteolysis. The first clue to this cell-cell interaction is that LH has little effect on P4 production in the separated LC and SC (Buhr, 1987; Agu, 1991; MSI), but LH increased P4 production in mixed cells (Wiesak and Foxcroft, 1992). The second indication is that mitochondria are increased predominantly in the SC but SER is primarily increased in the LC (ß-LC) (MSI). Initial P4 synthesis occurs in the mitochondria, and then the synthesized products (pregnenolone) are transferred to SER and converted to P4 (Niswender and Nett, 1988). It is therefore speculated that $PGF_{2\alpha}$, and maybe oxytocin, initiate luteolysis through the SC (MSI). In turn, the LC become more sensitive to $PGF_{2\alpha}$ and oxytocin which induces complete luteolysis of the CL through the PKC system. Further work is needed to investigate the influence of $PGF_{2\alpha}$ and oxytocin on PKC isozymes at different times during the estrous cycle and to demonstrate a link or signal between LC and SC. Thereby, it would be possible to find a more accurate regulating mechanism for P4 production in porcine CL.

PMA treatment resulted in the depressed P4 production at 12 and 24 h in the LC and SC from both days 10 and 15 (MSII and MSIII), but the LC still had membrane-bound PKC activity. Both LC and SC had cytosolic PKC deficiency (MSII). It is not clear what the relationship is between membrane-bound PKC and P4 production. But, at least, the membrane-bound PKC does not

enhance P4 production since reincubation of PKC deficient LC with PMA did not increase P4 production on day 10, and only moderately increased P4 production in PKC deficient SC (MSII). The SC may have more intracellular PKC that is not readily measurable in the assay used (MSII). It is therefore possible that the depressed P4 production on day 10 in response to long term reincubation with PMA may result from cytosolic deficiency of PKC. Another possibility is that PKC activity may shift from stimulatory to inhibitory from 2 to 12 and 24 h by unknown mechanisms. On day 15, activation of PKC depressed P4 production from 2 h to 12 and 24 h, indicating that within the first 2 h of incubation, at least, the response to PKC activation is distinctly different on day 10 and day 15.

PMA and 4α-phorbol did not influence testosterone production by the LC and SC on days 10 and 15. This indicates that PKC may limit its phosphorylative effect to the enzymes which are related to P4 production. It has been suggested that activation of protein kinase A and/or PKC did not influence $P450_{scc}$ and 3β-HSD (Wiltbank et al., 1991). However, studies of gene expression of these two enzymes have shown that activation of PKC can involve these two enzymes (Li et al., 1992). The current work provides evidence that activation of PKC influences P4 but not testosterone and 17β-estradiol production in isolated porcine luteal cells.

That PKC was primarily involved in the differential

response from days 10 and 15 is further supported by the results with 4α-phorbol and H-7. 4α-phorbol, a phorbol ester which does not activate PKC, did not influence P4 production on days 10 and 15. H-7 blocked the stimulatory effect of PMA on day 10 and the inhibitory effect on day 15 in short-term incubation. H-7 was demonstrated to inhibit PKC activity specifically by blocking protein phosphorylation by inhibiting ATP binding to protein kinase (Goodman et al., 1990). A short-term activity (MSII) of H-7 could result in the lack of an H-7 influence on P4 production in PMA+H-7 group at 12 and 24 h incubation.

In summary, activation of PKC has distinctly differential effects on P4 production during the luteal period: stimulatory in the middle of luteal phase but inhibitory in the late luteal phase. Depressed P4 production at 12 and 24 h on day 10 may be due to functional PKC deficiency. Conversely, PKC activation may shift from a stimulatory effect to an inhibitory effect on P4 production. Activation of PKC does not cause any change in testosterone and 17ß-estradiol by the isolated porcine luteal cells on both days 10 and 15. $PGF_{2\alpha}$, and possibly oxytocin, appears capable of stimulating P4 production in the middle of the luteal phase but inhibiting P4

production in the late luteal phase conceivably through the

PKC enzyme system.

Figure 1. Incubation times and arrangements for studying the effect of PMA on progesterone production in the porcine large and small luteal cells on days 10 and 15 of the estrous cycle

Luteal Cells (LC & SC)

Day 10   Day 15

Control, 10 μM PMA   Control, 1, 10 and 50 μM PMA

| | | | | | |
0 30 60 90 120 150 180
        (min)

                                                  2 H

12 and 24 H   12 and 24 H

Figure 2. Effects of 10 μM PMA, 4α-phorbol ester, H-7 and PMA+H-7 on progesterone production (ng/3X10^4 cells, LSM ± sem) during the first 3 h incubation in the large cells on day 10 during the estrous cycle (n = 6). The samples were collected at 30 minute intervals. * (P < 0.05) different from 0 μM level.
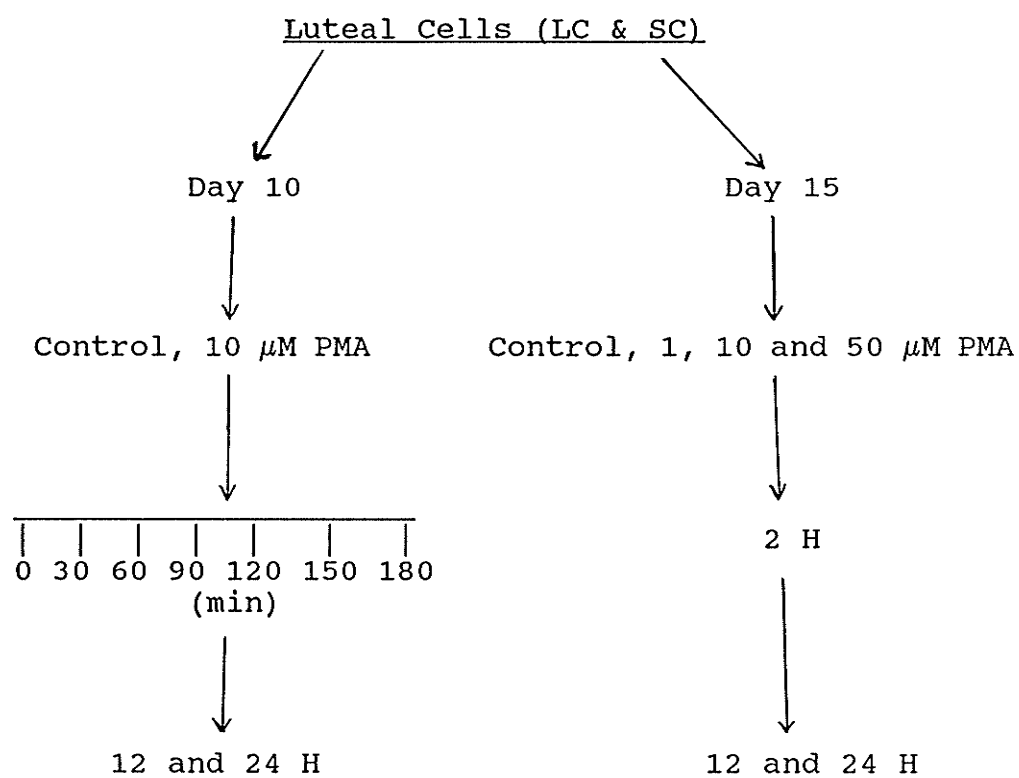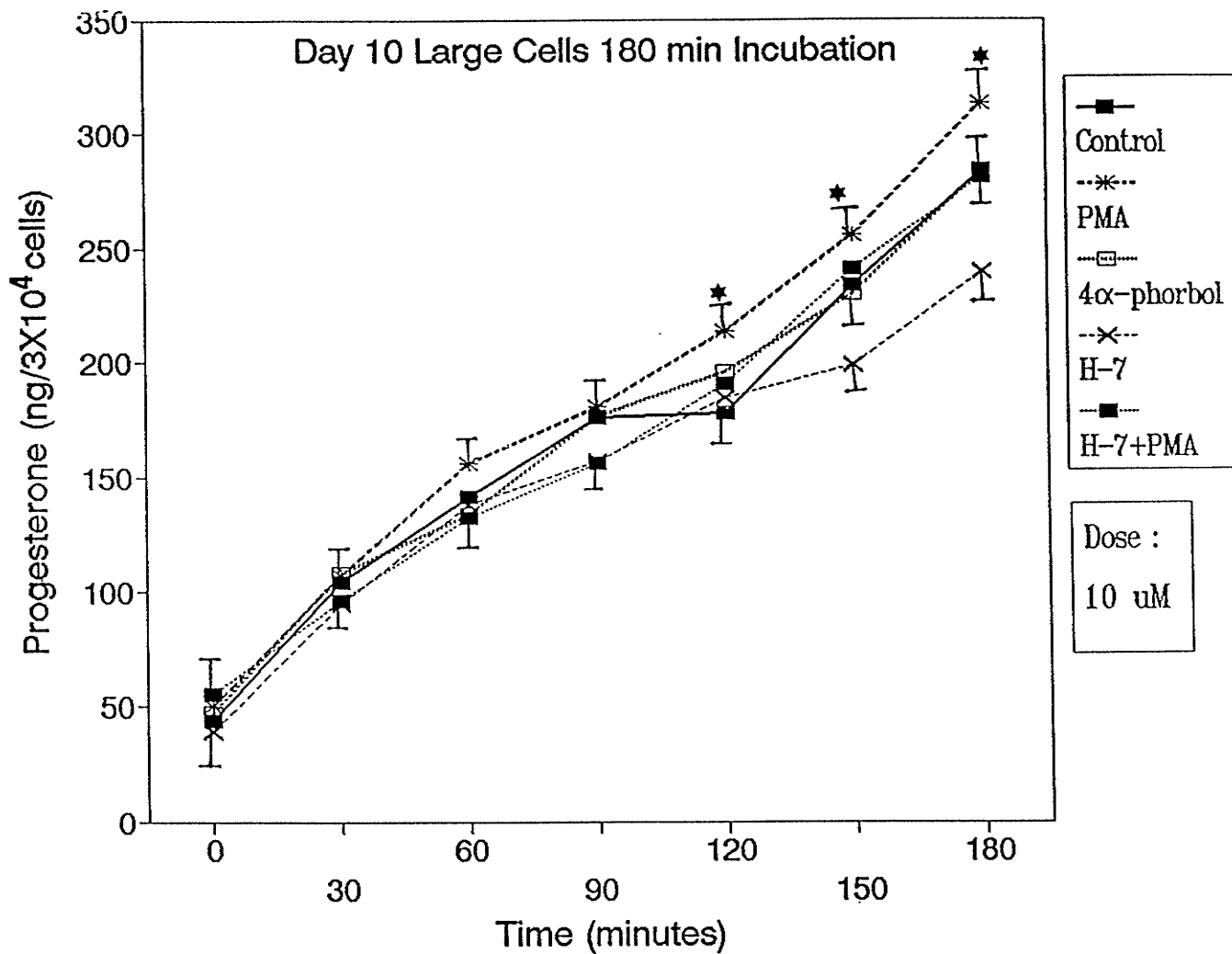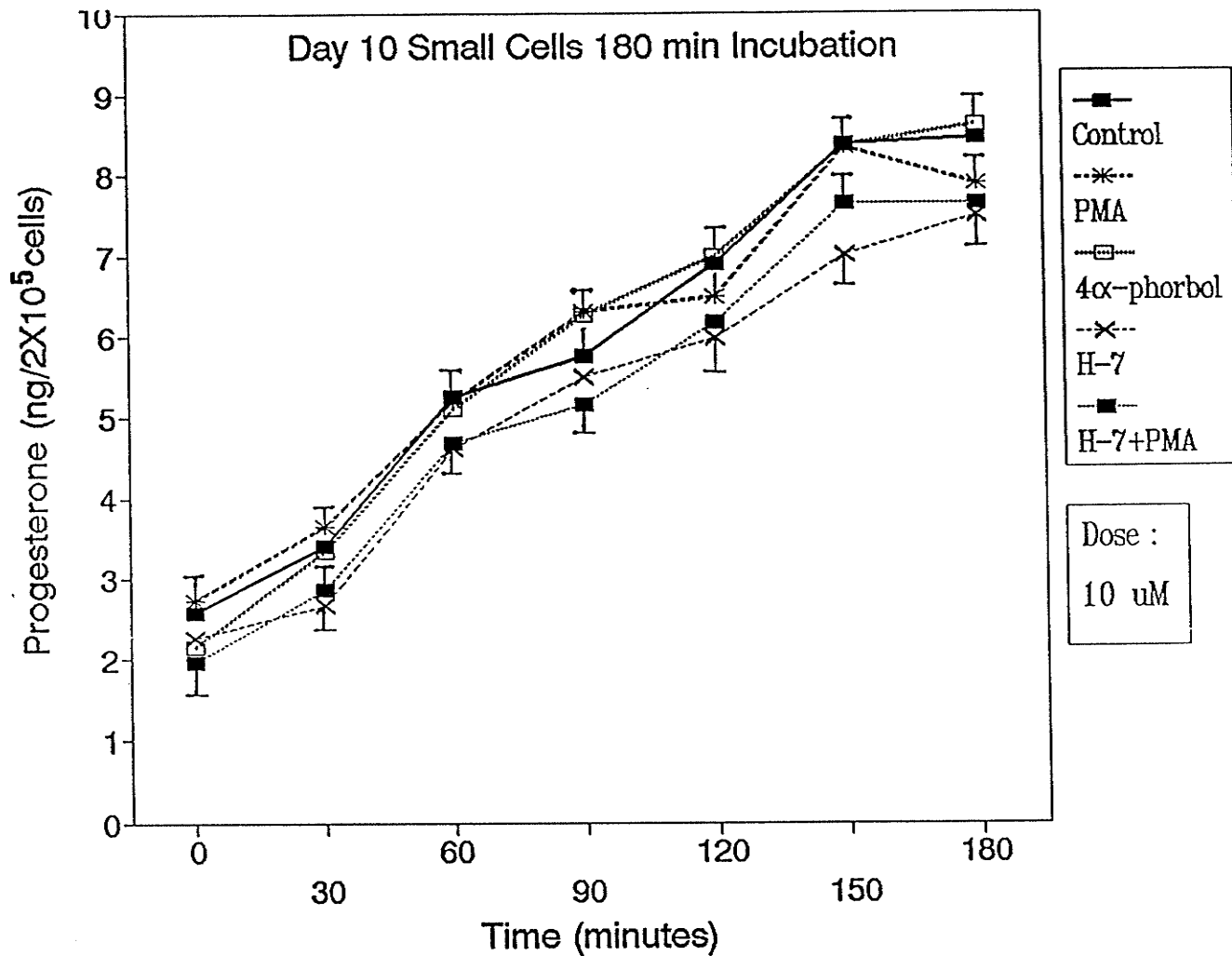
Figure 3. Effects of 10μM PMA, 4α-phorbol ester, H-7 and PMA+H7 on progesterone production (ng/2X10^5 cells, LSM ± sem) during the first 3 h incubation in the small cells on day 10 during the estrous cycle (n = 6). The samples were collected at 30 minute intervals. * (P < 0.05) different from 0 μM dose of PMA.
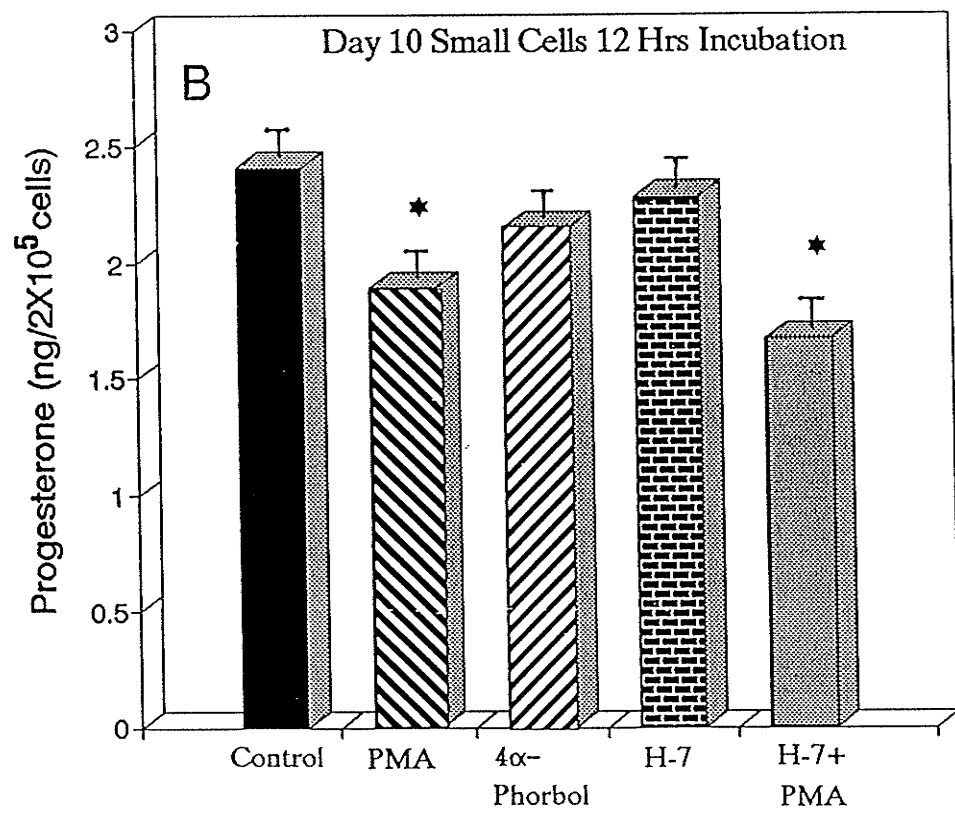
Figure 4. Effects of 10 μM PMA, 4α-phorbol ester, H-7 and PMA+H-7 on progesterone production (ng/3X10^4 large cells and ng/2X10^5 small cells, LSM ± sem) at 12 h incubation in the large (A) and small (B) cells on day 10 during the estrous cycle (n = 6). * (P < 0.05) different from 0 μM dose of PMA.

Figure 5. Effects of 10 μM PMA, 4α-phorbol ester, H-7 and PMA+H-7 on progesterone production (ng/3X10^4 large cells and ng/2X10^5 small cells, LSM ± sem) at 24 h incubation in the large (A) and small (B) cells on day 10 during the estrous cycle (n = 6). * (P < 0.05) different from 0 μM dose of PMA.

Day 10 Large Cells 180 min Incubation

Day 10 Small Cells 180 min Incubation

A. Day 10 Large Cells 12 Hrs Incubation

Progesterone (ng/3X10^4 cells) vs Control, PMA, 4α-Phorbol, H-7, H-7+PMA

B. Day 10 Small Cells 12 Hrs Incubation

Progesterone (ng/2X10^5 cells) vs Control, PMA, 4α-Phorbol, H-7, H-7+PMA

Figure A. Day 10 Large Cells 24 Hrs Incubation

Figure B. Day 10 Small Cells 24 Hrs Incubation
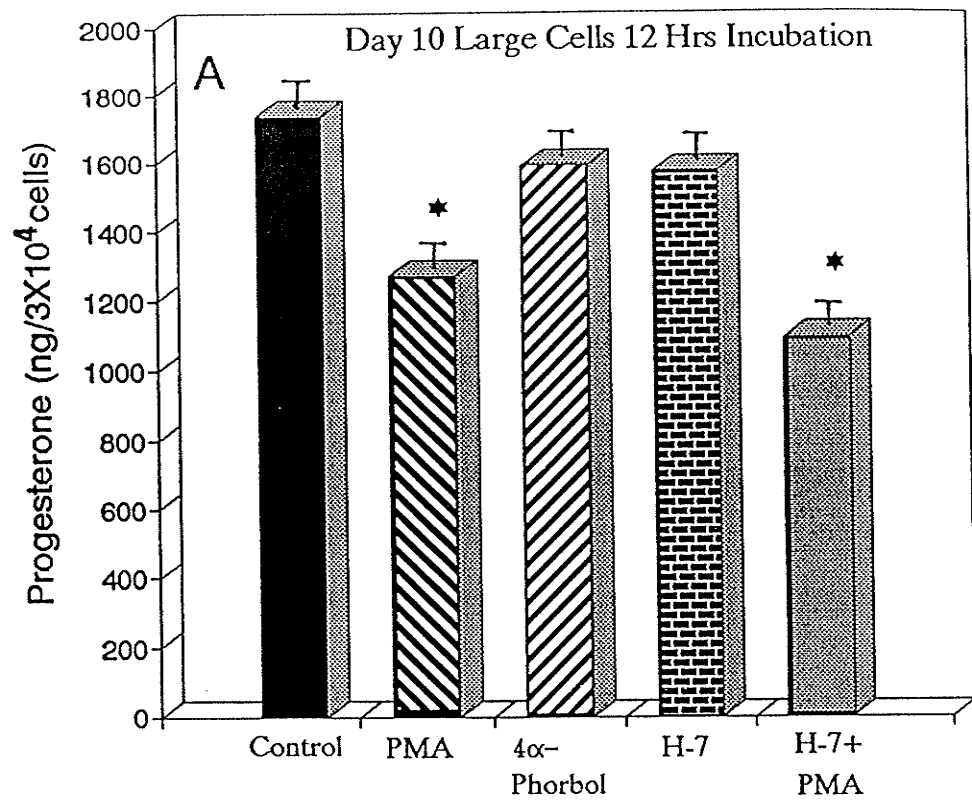
Figure 6. Effects of $10 \mu M$ PMA, 4α-phorbol ester, H-7 and PMA+H7 on progesterone production (ng/$3X10^4$ large cells and ng/$2X10^5$ small cells, LSM ± sem) at 2 h incubation in the large (A) and small (B) cells on day 15 during the estrous cycle (n = 4). * (P < 0.05) different from 0 μM dose of PMA.

Figure 7. Effects of 10 μM PMA, 4α-phorbol ester, H-7 and PMA+H-7 on progesterone production (ng/$3X10^4$ large cells and ng/$2X10^5$ small cells, LSM ± sem) at 12 h incubation in the large (A) and small (B) cells on day 15 during the estrous cycle (n = 4). * (P < 0.05) different from 0 μM dose of PMA.

Figure 8. Effects of 10 μM PMA, 4α-phorbol ester, H-7 and PMA+H-7 on progesterone production (ng/$3X10^4$ large cells and ng/$2X10^5$ small cells, LSM ± sem) at 24 h incubation in the large (A) and small (B) cells on day 15 during the estrous cycle (n = 4). * (P < 0.05) different from 0 μM dose of PMA.
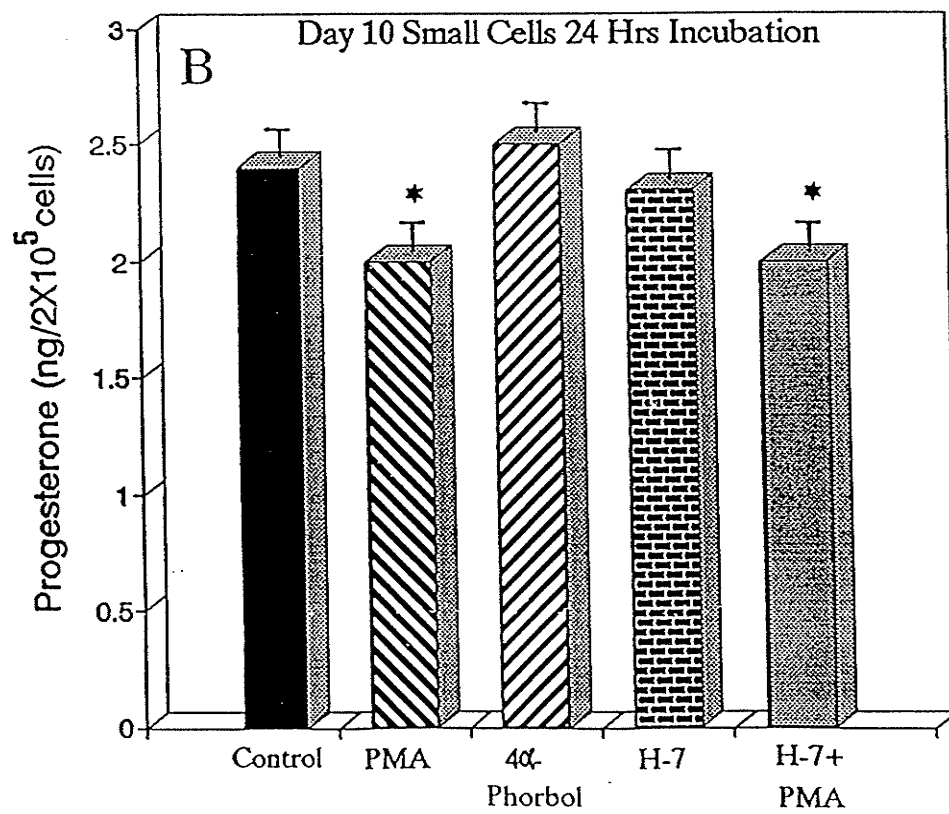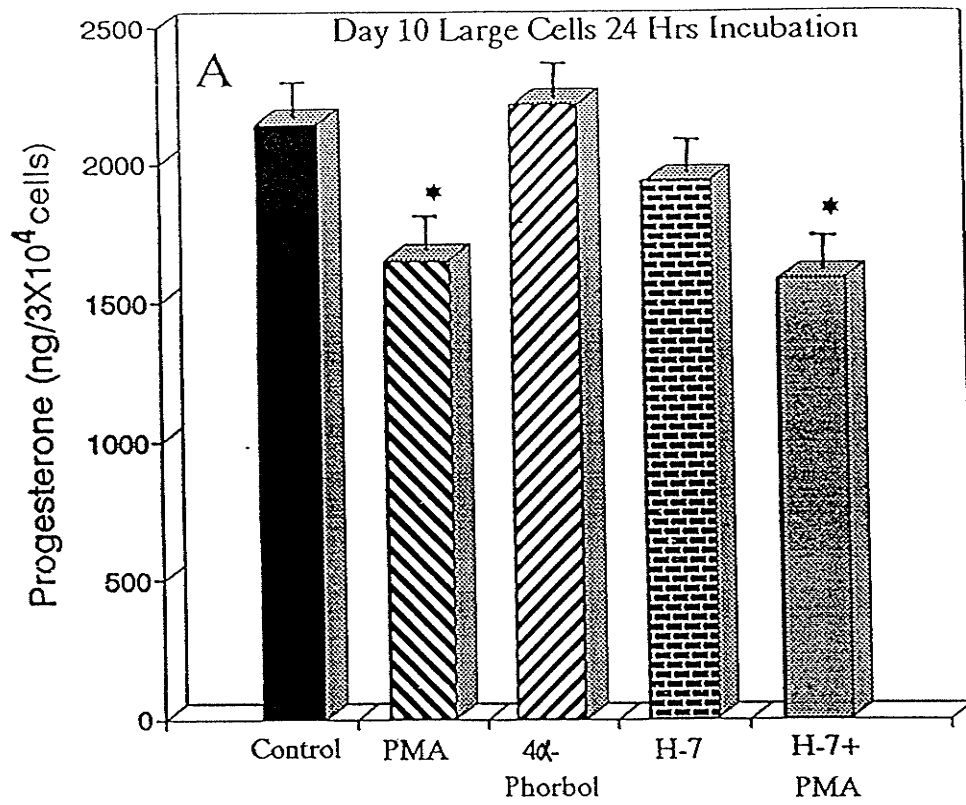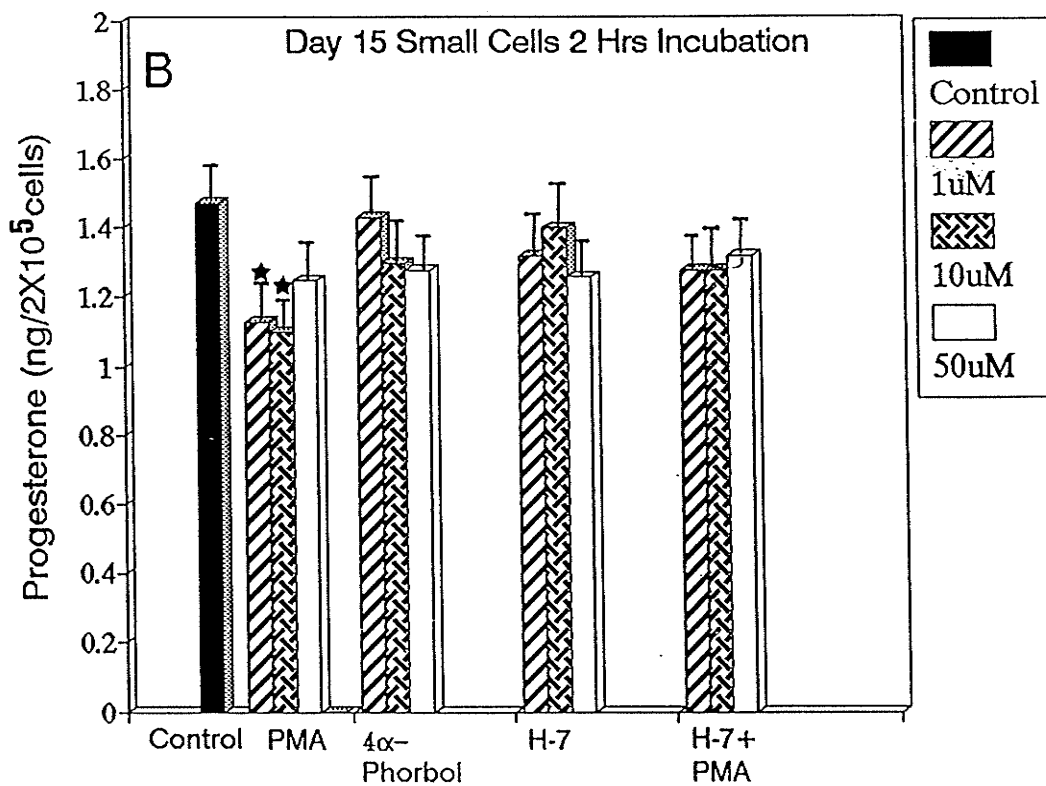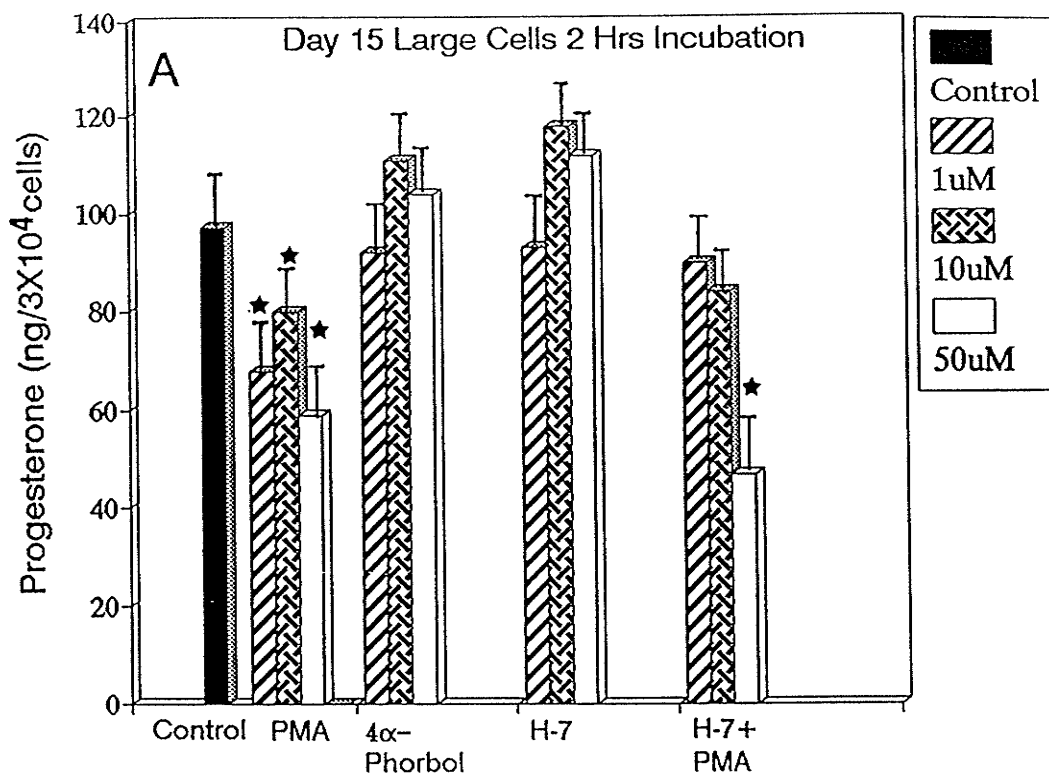
A Day 15 Large Cells 2 Hrs Incubation

Progesterone (ng/3X10^{4}cells)

Legend: ■ Control, ▨ 1uM, ▧ 10uM, □ 50uM

x-axis: Control, PMA, 4α–Phorbol, H-7, H-7+ PMA

B Day 15 Small Cells 2 Hrs Incubation

Progesterone (ng/2X10^{5}cells)

Legend: ■ Control, ▨ 1uM, ▧ 10uM, □ 50uM

x-axis: Control, PMA, 4α–Phorbol, H-7, H-7+ PMA

A Day 15 Large Cells 12 Hrs Incubation

Progesterone (ng/3X10^{4}cells)

Control | PMA | 4α–Phorbol | H-7 | H-7+ PMA

Legend: Control, 1uM, 10uM, 50uM

B Day 15 Small Cells 12 Hrs Incubation

Progesterone (ng/2X10^{5}cells)

Control | PMA | 4α–Phorbol | H-7 | H-7+ PMA

Legend: Control, 1uM, 10uM, 50uM

A Day 15 Large Cells 24Hrs Incubation

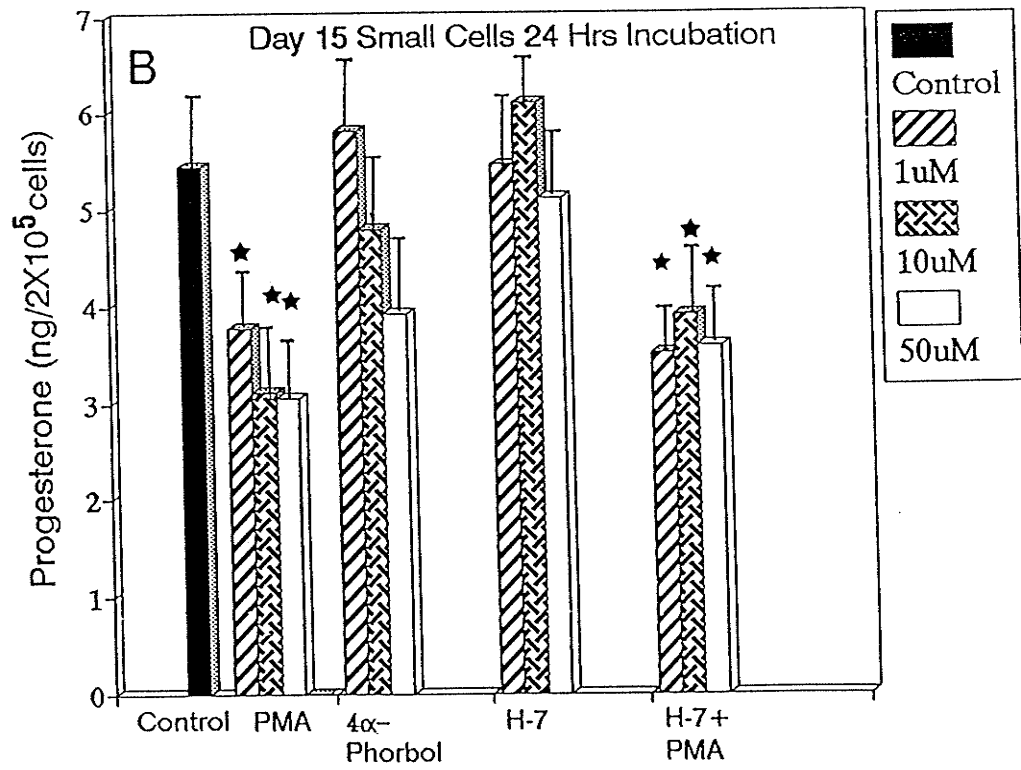**B** Day 15 Small Cells 24 Hrs Incubation

Table 1. The effect of 10 μM PMA and 4α-phorbol ester on testosterone production by large and small porcine luteal cells from days 10 and 15 of the estrous cycle at 2, 12 and 24 h incubation [LSM±sem,ng/3X10^4(Large) or /2X10^5 cells(Small)].

			Incubation time (h)		
treatment	day	cell size	2	12	24
control	10	large	4.0±0.5	9.2±0.4	9.8±0.5
PMA	10	large	4.3±0.4	10.3±0.5	11.2±0.4
4α-phorbol	10	large	3.7±0.5	8.3±0.5	8.6±0.5
control	10	small	0.7±0.08	1.4±0.1	1.5±0.1
PMA	10	small	0.8±0.1	1.2±0.09	1.3±0.09
4α-phorbol	10	small	0.9±0.09	1.6±0.1	1.8±0.1
control	15	large	4.5.0±1.1	10.3±1.0	11.6±1.1
PMA	15	large	3.8±1.0	10.0±1.1	10.4±1.0
4α-phorbol	15	large	3.9±1.1	11.3±1.0	9.7±1.1
control	15	small	1.4±0.06	2.3±0.07	2.3±0.06
PMA	15	small	1.2±0.07	2.2±0.06	2.2±0.06
4α-phorbol	15	small	1.3±0.06	2.2±0.06	2.5±0.07

GENERAL DISCUSSION

The Fine Structure and P4 Production by the LC and SC

The fine structure of luteal cells of cattle, sheep and pigs have similarities but also distinguishing differences. LC from bovine (Hansel et al., 1987), sheep (Niswender and Nett, 1988) and porcine CL (Yuan and Connor, 1990b) have abundant mitochondria, SER, extensive microvilli projections and highly convoluted membrane surfaces. However, the shape and size of SER in the porcine LC are extremely different from the bovine and ovine. The primary distinguishing feature in the porcine LC was large, fingerprint and tubular SER, which occupied about 40% of the cellular area (MSI). This abundance and arrangement of SER is not found in the bovine and ovine LC but is confined to the SC (Hansel et al., 1987). Interestingly, not all the LC from the porcine CL contained this kind of SER. Approximately 35% of LC had large, fingerprint and tubular SER whereas 65% of LC contained small or non-tubular-shaped SER on day 10. Patterned after the classification in the bovine (Fields et al., 1991) the cells with the small, nontubular shaped SER may be referred to as alpha LC and the other type as beta LC. The number of beta LC increased to 53% on day 30 and 66% on day 60 of pregnancy (MSI). It appears that the number of beta LC increases when the cyclic CL develops into the pregnant CL, which indicates that alpha LC can develop

into beta LC.

SER is extremely important for P4 synthesis. Cholesterol is first transported to the mitochondria, where it is converted to pregnenolone by $P450_{scc}$. The pregnenolone is transported to the SER and is converted to P4 by a key enzyme, 3ß-HSD (Niswender and Nett, 1988). The different kind of SER in alpha and beta LC may be related to the responsiveness of porcine luteal cells to $PGF_{2\alpha}$ in vivo and in vitro. For example, although the bovine alpha and beta LC do not contain similar fingerprint SER (Field et al., 1991) as in the porcine LC, the beta bovine LC, which were more sensitive to $PGF_{2\alpha}$ and underwent regression first (Fields et al., 1991) had numerous mitochondria and a more apparent SER than small alpha LC. It is well known that porcine CL in the mid luteal phase are unresponsive to $PGF_{2\alpha}$ (Connor et al., 1976). This may be because the majority of cells are the alpha LC type that are unresponsive to the luteolytic effect of $PGF_{2\alpha}$ in the mid luteal phase. However, in the late luteal phase, the predominant beta LC may make the porcine CL become sensitive to $PGF_{2\alpha}$ luteolysis.

The SC from bovine, ovine and porcine CL of the estrous cycle are also different. The bovine SC contained the fingerprint SER (Hansel et al, 1987), and similarly ovine SC also had large quantities of SER (Niswender and Nett, 1988). The porcine SC contained only a small amount of SER. The function of the fingerprint SER in the SC is not clear but the

SC show species differences in response to exogenous hormone treatment (MSI; Hansel et al., 1987; Niswender and Nett, 1988). As well, it has been demonstrated that SC produces much less P4 than LC in both ruminant and porcine CL (Wiltbank et al., 1991; MSI). This lower P4 production may be associated with the fewer microvilli, less SER and present in these cells as noted in the pig (MSI).

Hormonal Regulation of P4 Production by Porcine Luteal Cells

LH is generally considered as a luteinizing hormone in vivo since it stimulates the luteinization of follicular cells and promotes P4 production in CL (Niswender and Nett, 1988). However, when bovine and ovine luteal cells are incubated with LH, only SC, but not LC, are strongly stimulated (Hansel et al., 1987; Niswender and Nett, 1988). The porcine CL is insensitive not only to LH but also to $PGF_{2\alpha}$ luteolysis during the mid stage of the cycle (Connor et al., 1976). In vitro separated LC and SC from cyclic porcine CL were insensitive to LH (Hunter, 1981; Buhr, 1987; Agu, 1990; MSI). But when mixed porcine luteal cells from the cycle or pregnancy were used, LH produced either a minimal (Hunter, 1981) or a strong (Mattioli et al., 1985; Li et al., 1991; Wiesak and Foxcroft, 1992) stimulation. If LH stimulates P4 production by the mixed luteal cells, this would suggest that there is an important

link between LC and SC (Connor, personal communication, 1990), which is essential for optimal P4 production and responsiveness to LH. This proposal has not yet been confirmed.

FSH is a well known for its stimulation of follicle growth during the follicular phase of the estrous cycle but the role of FSH in the luteal phase is poorly understood. FSH receptors have only been detected in rat (Oxberry and Greenwald, 1982) and bovine (Manns et al., 1984) luteal cells. FSH receptors increased in the early and late luteal phase in the bovine CL (Manns et al., 1984). FSH receptors were not detected in porcine luteal tissues at unspecified days of the mid luteal phase (Ziecik et al., 1988). FSH stimulated P4 production by rat and bovine ovary tissues (Romanoff, 1966; Adashi et al., 1981; Barano and Hammond, 1985). Hansel and Seifart (1967) reported that FSH did not have any effect on P4 production in bovine luteal cells. FSH had little effect on P4 production by the porcine luteal cells on day 10 (MSI) nor in another study using mixed luteal cells which did not respond to FSH on days 8 to 10, and 12 to 14 after ovulation (Gregoraszczuk, 1992). However, in the current work, FSH inhibited P4 production by LC on day 15 in a dose dependent manner (MSI). Similarly FSH inhibited P4 production by porcine granulosa cells from large follicles (Hylka and diZerega, 1990). It is possible that FSH stimulates follicle development while it inhibits P4 production from the CL during a specific

time. The inhibitory effect on P4 secretion could be of benefit to follicular growth during this period.

Oxytocin and vasopressin are not only found in the brain but also in the ovary, the male gonad and accessory organs, the adrenal and the placenta (Hadley, 1988). The physiological role of these hormones from the various glands is still unknown (Fuchs, 1988). Similarly, the role of oxytocin in ovarian function has been widely studied but, as yet, is still poorly understood. In vivo, oxytocin has been reported to have a luteolytic function (Fuchs, 1988). However, the studies in vitro have been conflicting. Oxytocin had little effect on P4 production by rat, human and bovine luteal cells (Mukhopadhyay et al., 1984; Richardson and Masson, 1985; Hansel and Dowd, 1986). However, the effect was dose dependent in that at a low concentration oxytocin stimulated P4 production by bovine luteal cells and at higher doses was inhibitory (Tan et al., 1982; Tan and Biggs, 1984). In the current study, oxytocin tended to increase P4 production by LC at both 2 and 24 h incubation on day 10 of the estrous cycle (MSI). Interestingly, oxytocin decreased P4 production by day 15 LC at 2 h incubation and by the SC at 24 h incubation. As well oxytocin inhibited P4 production in mixed, dispersed porcine luteal cells from day 13 of the estrous cycle (Przala et al., 1986).

$PGF_{2\alpha}$ seems to have the potential for both luteotropic and

luteolytic effects in the porcine CL. $PGF_{2\alpha}$ in the current work tended to increase P4 production by LC on day 10 of the estrous cycle (MSI). As well, $PGF_{2\alpha}$ enhanced P4 production in dispersed porcine luteal cells from the mid luteal phase (Mattioli et al., 1985) and from early pregnancy (Wiesak, 1991). The stimulatory effect of $PGF_{2\alpha}$ on P4 production has also been noted in the small bovine luteal cells on days 10 to 12 of the estrous cycle (Alila et al., 1986; Alila et al., 1988; Benhaim et al., 1987). In contrast, $PGF_{2\alpha}$ at 10^3 pg inhibited P4 production at 2 h incubation by the LC on day 15 of the estrous cycle. At all doses $PGF_{2\alpha}$ inhibited P4 production by day 15 SC throughout the incubation period (MSI). Endogenous $PGF_{2\alpha}$ secretion is elevated on days 12 to 17 in cycling pigs and $PGF_{2\alpha}$ may act to up-regulate its own receptors on luteal cells in cycling pigs (Gadsby et al., 1992). It is suggested from both in vitro and in vivo studies that the porcine CL becomes sensitive to luteolysins such as $PGF_{2\alpha}$ only in the late luteal phase. It has been reported that repeated administration of $PGF_{2\alpha}$ made porcine CL become susceptible before day 12 of the estrous cycle (Estill and Gadsby, 1992). Perhaps such treatment serves to induce $PGF_{2\alpha}$ receptors and of itself does not explain the physiological luteolysis in pigs.

Our observation of the strong inhibition of P4 production by $PGF_{2\alpha}$ and oxytocin on day 15 may provide a unique insight into the process of luteolysis. Luteolysis in the late luteal

phase may be initiated in the SC. Agu (1990) found that the SC only became sensitive to perturbants of membrane phospholipid methylation, this is consistent with altered membrane fluidity associated with luteolysis in other species (Buhr et al., 1979; Carlson et al., 1982; Sawada and Carlson, 1991). Our observation of morphological dissimilarities of CL on day 15 further supported this proposal of a differential luteolysis between LC and SC. Mitochondria and lipid droplets were still obvious in LC but had almost disappeared in SC indicating a more advanced stage of luteolysis. It seems that P4 production by LC may depend on SC (Lemon and Mauleon, 1982; Wiesak and Foxcroft, 1992). If P4 production by LC depends on SC then the initial luteolysis in the SC would be a logical prelude to CL regression.

PKC Activity and Its Influence on P4 Production on Day 10 of the Estrous Cycle

It has been suggested that P4 production in LC is not regulated by cAMP systems although these cells contain adenylate cyclase (Hoyer et al., 1984). The bovine and ovine LC are not sensitive to LH (Hansel et al., 1987; Niswender and Nett, 1988). In contrast, the SC show a luteotropic response, and the increased P4 production in SC is proposed to be mediated by the PKA system in the ovine (Niswender and Nett, 1988) or through the PKC system in the bovine (Hansel et al.,

1987). Although LC produce much more P4 than SC (MSI) and occupy approximately 70% of the area in the CL (Parry et al., 1980), the detailed luteotropic mechanisms in LC, as well in SC, are not clearly understood. Recently the PKC second messenger systems have been implicated in the control of P4 production in ovarian tissue of various species (Hansel et al., 1987). There is greater PKC activity in the porcine CL but greater PKA activity in the follicles (Noland and Dimino, 1986). Furthermore, as follicular cells differentiate into large luteal cells , they develop a high capacity for P4 production, which does not appear to be regulated by the PKA pathway (Hoyer and Niswender, 1986). Therefore, we initially postulated that PKC may play a luteotropic role at the early and mid luteal phase in the porcine CL.

PMA, which is a well demonstrated PKC activator in CL (Noland and Dimino, 1986; Wheeler and Veldhuis, 1987), was used in our work. PMA stimulated P4 production by the LC and SC in short term incubation. By 12 and 24 h, P4 concentrations in PMA treated cells were depressed with the LC (MSII). In the bovine, low doses of PMA stimulated P4 production by SC on days 10 to 12 of the estrous cycle (Benhaim et al., 1987; Alila et al., 1988; Benhaim et al., 1990). Paradoxically an inhibitory effect of PMA on P4 production was reported in the ovine LC and LH-stimulated SC on day 10 of the estrous cycle (Wiltbank et al., 1989). It is therefore likely that factors such as species, cell type,

incubation time and presence of luteotropic agents affect the responses of cells to phorbol esters.

It has been proposed that a number of hormones depend upon the PKC system for their intracellular actions, which may be stimulatory or inhibitory (Nishizuka, 1986). LH is generally considered to play a luteotropic role in CL but LH has been demonstrated not to have the leutrotropic effect in ovine and bovine LC (Hansel et al., 1987; Niswender and Nett, 1988), which occupy most of the area of the CL and produce the greatest amount of P4 (Hoyer and Niswender, 1984). The porcine CL of the mid luteal phase is suggested to require little if any luteotropic support (Hunter, 1981; Buhr, 1987; Agu, 1990). Both the LC and SC from the porcine CL demonstrated minimal responsiveness only to high levels of LH after long term (24 h) incubation (MSI). Activation of PKA with forskolin in ovine LC increased cAMP production but not P4 production (Niswender and Nett, 1988). The results cited above indicate that cAMP and the PKA second messenger system does not work to regulate P4 in the LC.

What could be the hormonal link between enhanced PKC activation and corresponding P4 production in the porcine CL? One possibility is $PGF_{2\alpha}$. The $PGF_{2\alpha}$ affected a transient increase in P4 production in the porcine LC on day 10 (MSI), and from the bovine SC (Benhaim et al., 1987; Alila et al., 1988a; Alila et al., 1988b). As well $PGF_{2\alpha}$ stimulated basal or LH-treated P4 production in mixed porcine luteal cells

(Mattioli et al., 1985). Recent studies have demonstrated that $PGF_{2\alpha}$ initiates the breakdown of PIP2 to DG and IP3 in the bovine (Davis et al., 1987b; Davis et al., 1988), ovine (McCann and Flint, 1987), human (Davis et al., 1989) and rat (Leung et al., 1986; Lahav et al., 1988) luteal cells. DG and IP3 are the natural activators of PKC. On day 10, the porcine luteal cells, especially LC, responded to $PGF_{2\alpha}$, oxytocin and an activator of PKC (PMA). Thus, $PGF_{2\alpha}$ appears capable of stimulating P4 production by the porcine luteal cells possibly via PKC, in the middle of the luteal phase.

A perplexing observation was that the total PKC activity in the LC treated with PMA for 12 to 16 h was still as high as that in the normal LC, even though PMA treated LC showed cytosolic deficiency of PKC activity. In the ovine luteal cells (Wiltbank et al., 1989) and other types of cells (Thomas et al., 1987), treatment with PMA for 20 to 60 min resulted in the translocation of PKC activity to the membrane, which is considered to be evidence of PKC activation (Bell, 1986; Witters and Blachshear, 1987). However, longer incubation with PMA led to a significant loss of PKC activity due to proteolytic degradation of the enzyme. This 'down regulation' apparently, however, does not occur in all cell types. For example, incubation with a PKC activator for 30 min did not result in a loss in total PKC activity in parietal yolk sac cells. Instead, there was a tight association of PKC activity with the plasma membrane (Kraft and Anderson, 1983). The

results from the current work indicate that PMA stimulation of PKC activity in the porcine LC, at least, involves translocation and binding of PKC to the plasma membrane where it remains, leaving the cell functionally PKC deficient. Thus, the depressed P4 production after 12 and 24 h incubation with PMA likely results from functional PKC deficiency rather than prolonged PKC activation (Yuan and Connor, 1992).

There was a significant difference in PKC activity between the LC and SC from the porcine CL in the current work. The total PKC activity remained similar in the LC on days 10 and 15. The cytosolic PKC activity was greater in the LC than the SC on days 10 and 15 of the estrous cycle (MSII), which is similar to that in sheep (Wiltbank et al., 1989). The SC situation was unique in that the measurable cytosolic PKC was greater than the total PKC activity. Wiltbank et al. (1989) reported the membrane-bound PKC activity in the ovine LC but they did not mention it in the SC. They did not report the difference of total PKC activity between LC and SC either. Because the total PKC activity was lower in the SC in the current work, this may imply that as in some other cells (Thomas et al., 1987), a large portion of PKC is associated with the intracellular membrane fraction of organelles such as the mitochondria or endoplasmic reticulum and is more sensitive to destruction by the Triton X-100 treatment required to solubilize membrane-bound PKC. The physiologic significance of these cell type differences in the porcine CL

is not clear. However, the LC and SC do respond differently, especially on day 15 when we have noted the SC were more advanced in luteolysis and had greater responsiveness than the LC to the luteolytic effects of $PGF_{2\alpha}$ and oxytocin.

PKC Has a Distinctly Inhibitory Effect on P4 Production on Day 15 of the Estrous Cycle

The reinvestigation (MSIII) of the effect of PMA on P4 production on day 10 supported the previous findings (MSII) that activation of PKC increases P4 production in porcine LC from the mid-cycle. Interestingly, activation of PKC had a distinctly opposite effect on day 15. PMA inhibited P4 production by the LC and SC at short-term incubation (2 h) as well as long-term incubation (12 and 24 h). The mechanism(s) responsible for this shift in PKC activity from being stimulatory on day 10 to being inhibitory on day 15 is not clear. One possibility may be the emergence of different PKC isozymes. For example, activation of PKC inhibits the cAMP-generating system in NCB 20 and NIH 3T3 cells in which the alpha isozyme is expressed, whereas activation of PKC enhances the responses of the cAMP-generating system in PC 12 cells which contain both alpha and gamma isozymes (Gusovsly and Gutkind, 1991). Seven isozymes in the PKC family in murine tumour cell lines (Mischak et al., 1991) and three distinct isoforms of PKC in porcine luteal cytosol have been reported

(Wheeler and Veldhuis, 1989.). The effects of these isozymes or isoforms of PKC in cells are not clear. Three isoforms of PKC in the porcine luteal cytosol may have distinct actions for initiating luteolysis.

It is apparent that the observed effects on P4 production in the porcine LC and SC on days 10 and 15 was specifically related to PKC activity, because the non-PKC activator, 4α-phorbol ester did not have any influence on P4 production. H-7, an inhibitor of PKC activity, blocked the effects of PMA on P4 production on day 10 and day 15 in the short term incubation. H-7 has been demonstrated to inhibit PKC activity specifically by blocking protein phosphorylation by inhibiting ATP binding to protein kinase (Goodman et al., 1990). It is therefore speculated that H-7 blocks PKC from transferring phosphate from ATP to P4 synthesis enzymes. The short activity of H-7 (MSII) may result in the ineffectiveness of H-7 on PKC in long term incubation.

PMA did not have any influence on testosterone and 17ß-estradiol production in the LC and SC on days 10 and 15. This suggests that activation of PKC only influences P4 production. However, it is not clear which steroidogenic enzyme is phosphorylated by PKC activation. In the ovine, it has been proposed that PKC acts before the P450$_{scc}$ (Wiltbank et al., 1989) as activation of PKA or PKC did not alter the activity of P450$_{scc}$ and 3ß-HSD (Wiltbank et al., 1991). However, the studies of gene expression provide other evidence. For

example, $PGF_{2\alpha}$ decreased levels of mRNA for 3ßHSD and $P450_{scc}$ (Hawkins et al., 1992; Li et al., 1992) in sheep and pigs.

In summary, large, fingerprint and tubular SER are reported for the first time in the porcine LC on day 10 of the estrous cycle. Those cells having small, nonfingerprint SER are referred as alpha LC and those having large, fingerprint and tubular SER as beta LC. The number of beta LC increase when the CL of the estrous cycle develops into the CL of pregnancy. The current study provides observations which distinguish the porcine CL from the ruminant CL. Different cellular features between LC and SC are that LC have numerous microvilli on the membrane and the microvilli consist of micronetworks, but SC contain fewerer microvilli and no micronetworks. Another feature is that LC contain predominantly SER but SC have primarily mitochondria. The number of mitochondria increases when the cyclic CL grows into the CL of pregnancy. Low P4 production in the SC may result from the small amount of SER and microvilli.

The current study shows for the first time that FSH inhibits P4 production by day 10 SC and day 15 LC. This effect may be of benefit to follicular development during the luteal phase and needs to be studied further. Oxytocin and $PGF_{2\alpha}$ strongly inhibited P4 production especially in the SC on day 15. TEM showed that mitochondria and lipid droplets are still seen in the LC but they disappear in the SC. This and the strong inhibition of P4 production by oxytocin and $PGF_{2\alpha}$ in day

15 SC suggest that luteolysis in the late luteal phase may be initiated in the SC.

Protein kinase C activity and its influence on steroidogenesis were determined for the first time in the separated porcine LC and SC on days 10 and 15 of the estrous cycle. Incubation of the LC and SC with PMA resulted in translocation of PKC activity to the plasma membrane in the LC. The SC are unique in that the detectable cytosolic PKC activity was greater than total PKC activity. This may suggest that a large portion of PKC is associated with the intracellular membrane fraction of organelles such as mitochondria and SER. Activation of PKC did not influence testosterone and 17ß-estradiol production in separated luteal cells during the estrous cycle. However, activation of PKC can increase P4 production on day 10 and decrease P4 production on day 15. Since $PGF_{2\alpha}$, oxytocin and PMA coincidentally stimulated P4 production in the mid luteal phase and inhibited P4 production in the late luteal phase, this suggests that $PGF_{2\alpha}$, and possiboly oxytocin, could regulate the function of porcine CL through the PKC system during the estrous cycle.

CONCLUSION

1. There were two kinds of large cells in the porcine CL. One contained small, nonfingerprint SER, the other one had large, tubular and fingerprint SER, which occupied approximately 40% of the area in the cell. The former were referred to as alpha LC and the latter as beta LC. Alpha and beta LC were for the first time reported in the porcine LC. The number of beta LC increased when the cyclic CL differentiated into the pregnant CL. This indicates that alpha LC can grow into beta LC.

2. The LC contained numerous microvilli consisting of micronetworks, the SC contained only a small amount of microvilli. The SC also had fewer SER in the CL of the cycle and pregnancy but predominantly larger mitochondria. The number of mitochondria in the SC increased with pregnancy. The SC produced much less P4 than the LC, which may be associated with less SER and microvilli.

3. FSH was, for the first time, found to inhibit P4 production by the LC on day 15 of the estrous cycle. This unique effect of FSH may be of benefit to follicular development. Oxytocin and $PGF_{2\alpha}$ tended to increase P4 production on day 10 but decreased P4 production by the LC, and particularly by the SC on day 15 of the estrous cycle.

4. TEM indicated that the LC still contained mitochondria and lipid droplets, but these had almost disappeared in the SC by day 15 of the cycle. This and the strong inhibition of P4 production by oxytocin and $PGF_{2\alpha}$ in day 15 SC suggest that luteolysis in the late luteal phase may be initiated in the SC.

5. The translocation of PKC activity to the plasma membrane occurred in the LC when PKC was stimulated by an activator (PMA). The SC was unique in that the detectable cytosolic PKC activity was greater than total PKC activity. The LC contained more PKC activity than the SC. This may be associated with the different influence of luteotropic and luteolytic hormones on P4 production.

6. PMA increased P4 production on day 10 but decreased P4 production on day 15. This indicates that activation of PKC has a different effect on P4 production; being stimulatory in the middle of the luteal phase but inhibitory in the late luteal phase.

7. Preincubation of the porcine luteal cells with PMA for 12 to 16 h left the LC unresponsive to reincubation with PMA and the SC only moderately responsive to low levels of PMA on day 10. This indicates that the predominant effect of activation of PKC in day 10 LC is to stimulate P4 production.

8. P4 production appeared to be specifically induced by the PKC activation because a nonactivator of PKC (4α-phorbol ester) did not influence P4 production. As well, the PKC inhibitor, H-7, blocked the effects of PKC.

9. PMA did not influence testosterone and 17ß-estradiol production. This indicates that PKC limits its effect on the enzymes related to P4 production other than testosterone and 17ß-estradiol synthesis enzymes.

10 $PGF_{2\alpha}$, oxytocin and PMA coincidentally stimulated P4 production in the mid luteal phase and inhibited P4 production in the late luteal phase, indicating that $PGF_{2\alpha}$ and possibly oxytocin, could play a dual role in regulating the function of the porcine CL during the estrous cycle.

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