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Cellular mechanisms responsible for development of sensitivity of the bovine corpus luteum to prostaglandin F2 alpha

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**Cellular Mechanisms Responsible for Development of Sensitivity of
the Bovine Corpus Luteum to Prostaglandin F2 alpha**

Madhusudan P. Goravanahally

**Dissertation submitted to the
Eberly College of Arts and Sciences at
West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy in
Biology**

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Abstract

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Madhusudan P. Goravanahally

Prostaglandin F2 alpha (PGF_{2α}) brings about regression of the bovine corpus luteum (CL). This luteolytic property of PGF_{2α} is used in beef and dairy cattle to synchronize estrus. A limitation of this protocol is an insensitivity of the early CL to luteolytic actions of PGF_{2α}. The mechanisms underlying this differential luteal sensitivity are poorly understood. Therefore the main objective of the current study is to understand the cellular mechanism of luteal insensitivity. The developing CL has a maximum number of PGF_{2α} receptors; therefore differences in signaling events might be responsible for luteal insensitivity. Hence differential gene expression at two developmental stages of CL, days 4 (D-4) and 10 (D-10) post estrus, might account for differences in signal transduction pathways associated with luteal sensitivity. For example, differential expression of protein kinase C epsilon (PKCε /PRKCE) and its ability to regulate PGF_{2α}-stimulated rise in intracellular calcium concentration have been proposed to be part of luteal resistance mechanism. Therefore the current study investigates the: 1) physiological role of PRKCE in regulating the ability of PGF_{2α} to inhibit progesterone synthesis, 2) role of PGF_{2α}-stimulated rise in intracellular calcium in progesterone inhibitory actions of PGF_{2α}, 3) differential expression of a large portion of the luteal transcriptome during its developmental transition from early to mature stage, and 4) role of differentially expressed CAMKK2 in acquisition of luteolytic sensitivity to PGF_{2α}. Down-regulation of PRKCE significantly reduced the ability of PGF_{2α} to inhibit LH-stimulated progesterone accumulation. A pharmacological increase in intracellular calcium concentration [Ca²⁺]_i significantly inhibited LH-stimulated progesterone accumulation irrespective of luteal developmental stage. More importantly, buffering the rise in [Ca²⁺]_i reduced the ability of PGF_{2α} to inhibit progesterone accumulation. Microarray analysis identified 167 genes that were expressed differentially (p < 0.05). These were categorized into genes involved in cell signaling (12%), steroidogenesis and metabolism (10.2%), protein degradation (5.3%), transcription regulation and DNA biosynthesis (18.5%), protein biosynthesis and modification (18.5%), extracellular matrix and cytoskeletal proteins (9.5%), antioxidant property (3%), miscellaneous (17%), and unknown functions (6%). In addition, the *in vivo* administration of PGF_{2α} increased the expression of a guanine nucleotide binding protein (G protein), beta polypeptide 1 (GNB1) in D-4 CL and calcium/calmodulin dependent kinase kinase 2, beta (CAMKK2) in D-10 CL. Furthermore, large and small luteal steroidogenic cells, known to be targets for actions of PGF_{2α} were demonstrated to be a cellular source for CAMKK2. More importantly, *in vitro*, a CAMKK2 inhibitor significantly reduced the ability of PGF_{2α} to inhibit progesterone accumulation. In summary, a developmental increase in PRKCE expression combined with its ability to regulate [Ca²⁺]_i and the availability of CAMKK2 to mediated the actions of rise in [Ca²⁺]_i might be important components of the mechanism rendering the bovine CL sensitive to PGF_{2α}.

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List of Abbreviations

α HSD - α -Hydroxysteroid Dehydrogenase

β HSD-beta -Hydroxysteroid Dehydrogenase/delta5, delta 4, isomerase

$[Ca^{2+}]_i$ – Intracellular Calcium Concentration

9K-PGR - 9-Keto-Prostaglandin Reductase

AA – Arachidonic Acid

AKR - Aldo-Keto Reductase

AngII - Angiotensin II

AP-1 – Activator Protein -1

CaM - Calmodulin

CAMKK2/CAMKK β - Ca^{2+} -Calmodulin Dependent Kinase Kinase 2, Beta

cAMP - Cyclic Adenosine Monophosphate

CL - Corpus Luteum

COX- Cyclooxygenase

cPLA2 – Cytosolic Phospholipase A2

CSF – Colony-Stimulating Factor

DAG – Diacylglycerol

DAX-1 - Dosage sensitive sex reversal; Adrenal hypoplasia congenital; X chromosome; gene 1

DDBX - Dihydrodiol Dehydrogenase 3

EDN1 - Endothelin-1

EDNR – Endothelin Receptor

eNOS - Endothelial Nitric Oxide Synthase

ER – Endoplasmic reticulum

Fas-L – Fas Ligand

FCS – Fetal Calf Serum

FP – Prostaglandin F2 receptors

GC – Granulosa Cells

GH - Growth Hormone

GNB - Guanine Nucleotide-Binding Protein (G Protein), Beta Polypeptide

GPCR – G-Protein Coupled Receptor

HDL-High Density Lipoprotein

HMG-CoA -3-Hydroxy-3-Methylgluaryl Coenzyme A

IF- Interferon

IGF-1 - Insulin-Like Growth Factor-1

IL – Interleukin

KCIP-1 - Kinase C Inhibitor Protein-1

kDa - Kilo Daltons

LDL - Low-Density Lipoprotein

LH- Luteinizing Hormone

LLCs - Large Luteal cells

MAPK - Mitogen-Activated Protein Kinase

MCP-1 - Monocyte Chemo-Attractant Protein-1

NAPDH - Nicotinamide Adenine Dinucleotide Phosphate

OATP- 12-Transmembrane Organic Anion Transporting Polypeptides

P4 – Progesterone

P-450_{scc} - Cholesterol Side-Chain Cleavage Cytochrome P-450

PBR - Peripheral-Type Benzodiazepine Receptor

PGDH - Prostaglandin-15 Dehydrogenase

PGES – PGE Synthase

PGES - Prostaglandin E Synthase

PGF_{2α}- Prostaglandin F2 alpha

PGFM - 13,14-Dihydro-15-Keto Prostaglandin F2 Alpha

PGHS - Prostaglandin G/H Synthase

PGT – Prostaglandin Transporter

PHFS - PGF Synthase

PKA - Protein Kinase A

PKA – Protein Kinase A

PKCI-1 - Protein Kinase C Inhibitor-1

PLC - Phospholipase C

PRKC/PKC – Protein Kinase C

PRKCE/PKCε – Protein Kinase C epsilon

PRL - Prolactin

PTGS2 - Prostaglandin-Endoperoxide Synthase 2

RACKs - Receptors For Activated Kinases

RIA – Radioimmunoassay

ROS – Reactive Oxygen species

S.C – Subcutaneous

SCP-1 - Sterol Carrier Protein

SiRNA – Small Interfering RNA

SLCs - Small Luteal Cells

StAR- Steroidogenic Acute Regulatory Protein

TNF – Tumor Necrosis Factor

UOP – Utero-Ovarian plexus

VEGF- Vascular Endothelial Growth Factor

YWHAZ - Tyrosine 3monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, zeta polypeptide

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like to thank my wonderful wife Sneha and family for all their blessings, support and inspiration.

Chapter I: Review of literature

I. History

It was Regnier de Graaf who first described that “globular bodies” appeared on rabbit ovary after coitus and remained there until after parturition [De Graaf R, 1643]. The term **corpora lutea**, meaning “yellow bodies” was first given by Marcello Malpighi [Malpighi M, 1689]. Prenant [Prenant LA, 1898] suggested that corpus luteum might produce substances that regulate pregnancy and act as a gland of internal secretion. Beard [Beard J, 1897] proposed that corpora lutea were responsible for inhibition of estrus and ovulation during pregnancy. In rabbit that the corpora lutea were required for implantation and maintenance of pregnancy was first demonstrated by Fraenkel [Fraenkel L, 1903]. Later, Corner and Allen [Corner GW and Allen WM, 1929] demonstrated that the alcoholic extract of corpora lutea from sows was capable of maintaining pregnancy in ovariectomized rabbits. Subsequently in 1934, four different groups of scientists isolated and purified a crystalline form of luteal factor [Allen WM and Wintersteiner O, 1934; Butenandt A et al, 1934; Hartmann H and Wettstein A, 1934; Slotta KH et al, 1934]. In the same year, Slotta *et al.* described the structural formula of luteal factor and named it as progesterone [Slotta KH et al, 1934].

II. Development of Corpus luteum

The corpus luteum (CL) is a transient endocrine gland formed from the ovulated follicle, and it secretes the primary steroid hormone progesterone. The development of CL is a complex process. The rate of growth of CL is very rapid and can be compared to the process of wound healing and tumor formation [Smith MF et al, 1994]. For example, the CL of cattle weighs an average of 3 g during Day 3 after ovulation, whereas it weighs on average of 5.1 g on Days 14 post-estrus [Fields MJ and Fields PA, 1996]. The pre-ovulatory surge of luteinizing hormone (LH) induces ovulation of a follicle followed by luteinization and differentiation of residual follicular cells [Lipner H, 1988; McClellan MC et al, 1975; Niswender GD and Nett TM, 1988]. The luteinization process is a transition of pre-ovulatory follicle into a highly vascular tissue capable of secreting large quantities of progesterone. The capacity to secrete high rates of progesterone is accomplished by increased expression of progesterone producing enzymes such as cholesterol side-chain cleavage cytochrome P-450 complex (P-450_{scc}) and 3β -

hydroxysteroid dehydrogenase/delta5, delta 4, isomerase (3beta -HSD), steroid acute regulatory protein (StAR), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) and decreased expression of the enzymes that convert progesterone to estrogens such as 17 -hydroxylase cytochrome P-450 and aromatase cytochrome P-450 [Bao B and Garverick HA, 1998]. Granulosa cells express low amounts of HDL prior to luteinization [O'Shaughnessy PJ et al, 1990]. However, there is an increased expression of mRNA for HDL-receptor after luteinization [Landschulz KT et al, 1996]. During differentiation and growth of CL, there is an extensive tissue remodeling including, intermixing and migration of endothelial cells, thecal cells and fibroblasts in such a way that they are in close proximity to one another. During initial development, the wall of the follicle collapses into folds [Pederson ES, 1951; Priedkalns J and Weber AF, 1968] and capillaries invade the developing CL which appears to be under the influence of angiogenic and mitogenic factors such as fibroblast growth factor [Gospodarowicz D et al, 1985], platelet-derived growth factor [Khachigian LM et al, 1996], insulin-like factor-1 [Suh DY et al, 1992], heparin binding growth factor [Grazul-Bilska AT et al, 1992], and vascular endothelial growth factor (VEGF) [Redmer DA and Reynolds LP, 1996]. Neovascularization is an important process that occurs during the CL development. It consists of breakdown of the basement membrane, migration and proliferation of pericytes and endothelial cells followed by the development of capillaries. Capillary lumina constitute about 22% of total CL weight and endothelial cells constitute approximately 50% of the total luteal cell population. Consequently, the rate of blood flow to the CL is approximately 6-10 ml/gm/min and rate of oxygen consumption per unit weight is six times greater than liver, kidney, or heart. Granulosa cells undergo hypertrophy without proliferation whereas the thecal and endothelial cells, and fibroblasts undergo mitosis and migration during the development of CL. For example, in sheep, from days 4 to 16 of estrous cycle fibroblasts approximately get double in number (from 21 to 50×10^6), whereas the number of endothelial cells increased by six-fold (18 to 120×10^6) [Farin CE et al, 1986].

The cells secreting progesterone are derived from differentiation of resident granulosa and thecal cells. The large luteal cells (20-30 μm) designated as LLCs are derived from granulosa cells and small luteal cells (<20 μm) designated as SLCs are derived from thecal cells [Alila HW and Hansel W, 1984]. During the development, the number of SLCs increases 5-fold (10 to 50×10^6), however size remains constant, whereas the size of LLCs increase approximately to two-fold while their number remains constant (15×10^6 / CL in sheep). Cellular hypertrophy during the

development is associated with an increase in cytoplasmic:nuclear ratio, increased number of smooth endoplasmic reticulum, increase in size of golgi apparatus, and increased number and complexity of mitochondria [Cavazos LF et al, 1969; Priedkalns J et al, 1968]. In terms of total cellular percentage of luteal tissue, LLCs constitute 4% and SLCs comprise 19% [Rodgers RJ et al, 1984]. It is important to note that LLCs make up 25% of the volume of the CL even though they add up to only 4% of luteal cell population. In addition to their morphological differences, they exhibit distinct biochemical differences and similarities. The basal amount of progesterone produced by LLCs is more than that produced by SLCs. For example, in monkey and sheep, large cells produce more than 10 times as much progesterone as produced by small cells, whereas human large cells produce twice as much as small cells [Ohara A et al, 1987]. In addition, LLCs do not respond to progesterone stimulatory action of LH, whereas SLCs respond to LH by increased production of progesterone [Fitz TA et al, 1982]. Immune cells such as plasma cells, lymphocytes and granular leukocytes form the important cellular components (7%) of CL in addition to steroidogenic luteal cells.

In ruminants, development and maturation of CL is under the influence of several hormones. For example in ruminants and primates, LH is the primary luteotropic hormone that supports the growth of CL. In primates [Fraser HM et al, 1986] and sheep [Kaltenbach CC et al, 1968] hypophysectomy caused regression of CL and this effect can be reversed by exogenous LH. In addition, pulses of LH are required for growth and development of fully functional CL in cattle, and to maintain secretion of progesterone during late luteal phase in sheep [Peters KE et al, 1994]. In dogs and cats, both LH and prolactin appear to be important for normal development of CL, however their specific requirements appear to differ with different stages of luteal development and pregnancy [Concannon PW et al, 2009].

In rodents, prolactin plays an important role in maintaining the function of the CL throughout the pregnancy. The important function of prolactin is to prevent premature expression of progesterone catabolizing enzyme, 20α -hydroxysteroid dehydrogenase (20α HSD). In addition, estradiol secreted by luteal cells has been shown to support the development of the CL by stimulating the synthesis of progesterone, vascularization and growth of CL [Stocco C et al, 2007]. In 1981, Rothchild [Rothchild I, 1981] proposed that progesterone secreted from CL might play an important protective role against regression. Accordingly, several investigators provided evidences to support this hypothesis. For example, in rats, administration of

progesterone antibody or progesterone receptor antagonist directly into the ovary inhibited the production of progesterone and administration of synthetic progesterone enhanced the synthesis of progesterone [Telleria CM and Deis RP, 1994; Telleria CM et al, 1999]. The ability of progesterone to auto-stimulate its own synthesis might be due to its ability to down-regulate the progesterone-metabolizing enzyme, 20 α -hydroxysteroid dehydrogenase (20 α -HSD) [Telleria CM et al, 1999]. In addition, progesterone inhibited luteal cell death by decreasing the expression of Fas [Kuranaga E et al, 2000]. In rodents, prolactin (PRL) has been shown to sustain the function of the CL by preventing the premature expression of 20 α -HSD [Grosdemouge I et al, 2003]. In agreement to Rothchild's hypothesis, in sheep progesterone might prevent the apoptosis of small luteal cells by preventing the ability of oxytocin to stimulate an increase in intracellular calcium [Niswender GD et al, 2007].

III. Synthesis of progesterone by luteal tissue

1. Source and substrate

Progesterone is the primary steroid hormone secreted by the bovine CL. However, pigs, rats, humans, cattle, and other species retain their ability to produce estradiol. The synthesis of progesterone is the least complex steroidogenic pathway in the ovary. Cholesterol is the starting substrate for the synthesis of P4. It has been shown that ovarian tissue preferentially utilizes lipoprotein-derived cholesterol rather than *de novo* synthesized cholesterol [Andersen JM and Dietschy JM, 1978]. However, luteal cells are capable of utilizing acetate as a source of cholesterol under the conditions of lipid deprivation [Cook B et al, 1967]. Most of the cholesterol for steroidogenesis in the CL is derived from low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [Hwang J and Menon KM, 1983; Ohashi M et al, 1982]. LDL is transported into the luteal cell via receptor-mediated endocytosis [Brown MS and Goldstein JL, 1986], where in the cell the endosome combines with lysosome to release free cholesterol and LDL receptor is recycled [Grummer RR and Carroll DJ, 1988]. HDL uptake into the cell occurs after binding to a plasma membrane-bound HDL binding protein; however, the exact mechanism of release and transport of cholesterol inside the cell is not clear [Lestavel S and Fruchart JC, 1994]. Once the free cholesterol is released inside the cell, it can be utilized in steroidogenesis or re-esterified with fatty acids by cholesterol ester synthetase and stored in the form of lipid droplets. Stored cellular cholesterol ester can be utilized as free cholesterol after being hydrolyzed by a

cholesterol esterase [Johnson WJ et al, 1997]. This step is hormonally controlled as cholesterol esterase is activated by protein kinase A (PKA)-dependent phosphorylation [Caffrey JL et al, 1979; Pittman RC et al, 1975].

2. Transport of cholesterol into the mitochondria

Cholesterol is transported to the inner mitochondrial membrane by a complex process. The transport of cholesterol from outer to inner mitochondrial membrane is the rate-limiting step during steroidogenic pathway. During this transport mechanism that peptide hormones and the secondary messenger cAMP accelerate the transport of cholesterol and enhance steroidogenesis [Miller WL, 1988; Simpson ER and Waterman MR, 1983]. The transport of cholesterol to outer mitochondrial membrane appears to be mediated by cytoskeleton [Crivello JF and Jefcoate CR, 1978] and sterol binding proteins [Scallen TJ et al, 1985]. Steroidogenic acute regulatory protein (StAR) [Stocco DM and Clark BJ, 1996] and peripheral-type benzodiazepine receptor (PBR) [Papadopoulos V, 1993] are involved in the transport of cholesterol from outer to inner mitochondrial membrane. StAR is initially formed as a 37 kDa and subsequently cleaved to inactive 30- and 32-kDa proteins upon transport to mitochondria. The X-ray crystal structure of StAR has homology to MLN64, a domain found in other lipid-transfer molecules, and can bind to sterol and facilitate the transfer of cholesterol from sterol rich unilamellar vesicles to acceptor membranes [Tsujishita Y and Hurley JH, 2000] [Kallen CB et al, 1998; Tuckey RC et al, 2002]. Supporting its role, addition of StAR to isolated mitochondria stimulated steroidogenesis [Bose H et al, 2002]. It has been proposed that cholesterol is being transported into the inner mitochondrial membrane during the insertion of StAR protein. Mutations in the StAR gene significantly reduced adrenal and gonadal steroid synthesis in patients affected with congenital lipoid adrenal hyperplasia, clearly suggesting the critical role of StAR in mitochondrial cholesterol transport. PBR is an 18 kDa protein and has high affinity for benzodiazepine diazepam [Gavish M et al, 1999; Papadopoulos V, 1993]. The ligands for PBR stimulated steroid synthesis in various cell types, and in isolated mitochondria [Lacapere JJ and Papadopoulos V, 2003; Papadopoulos V, 1993]. Greater concentrations of PBR have been reported to be located at outer/inner mitochondrial membrane sites [Culty ML et al, 1999] and it has high affinity to bind the cytosolic carboxy-terminal domain of the cholesterol [Lacapere JJ et al, 2001; Li H and Papadopoulos V, 1998]. Further, fluorescent resonance energy transfer

between StAR and PBR has indicated that these two molecules might interact such that StAR might transport the cholesterol to PBR in outer mitochondrial membrane [West LA et al, 2001].

3. Conversion of cholesterol to progesterone

The enzymes present in contact with the inner mitochondrial hydrophobic membrane, cytochrome P450 side-chain cleavage (P450_{scc}), and matrix enzymes such as adrenodoxin and adrenodoxin reductases catalyze the conversion of cholesterol to pregnenolone [Stone D and Hechter O, 1954]. This reaction consists of three steps, 20 α -hydroxylation, 22-hydroxylation, and cleavage of cholesterol side chain to yield pregnenolone and isocaproic acid. Each step of catalysis requires 3 molecules of nicotinamide adenine dinucleotide phosphate (NADPH). Adrenodoxin and adrenodoxin reductase enzyme help in transport of electron from NADPH to P450_{scc}.

Pregnenolone is then transported into smooth endoplasmic reticulum and converted into progesterone by an enzyme, 3-beta-hydroxysteroid dehydrogenase (3- β HSD) [Hanukoglu I, 1992; Stocco C et al, 2007]. This enzyme is a 42 kDa protein that has both dehydrogenase and isomerase activity in a single protein. Progesterone then appears to diffuse out of the cells as there is no evidence for its cellular storage. Corpora lutea of cattle secrete additional steroids such as 20beta-hydroxy-preg-4-en-3-one, and 20alpha-hydroxyl steroids.

4. Regulation of Progesterone synthesis

In most species, LH is the primary hormone involved in the regulation of progesterone synthesis and secretion from a CL. LH stimulated the synthesis of progesterone in sheep [Hoyer PB et al, 1984], cattle [Alila HW et al, 1988b], human [Ohara A et al, 1987], and pig [Tekpetey FR and Armstrong DT, 1991]. In primates, secretion of progesterone is dependent on the pulsatile secretion of LH throughout the luteal phase [Fraser HM et al, 1986], whereas in sheep, only basal amounts of LH secretion are sufficient to maintain the secretion of progesterone [McNeilly AS et al, 1992]. In cattle, it appears that only basal amounts of LH are required to maintain the secretion of progesterone during the later luteal phase, however the pulsatile secretion of LH is necessary for the development of CL [Peters KE et al, 1994]. In rodents, prolactin (PRL) produced from the pituitary gland in response to mating reflex has role in maintaining the secretion of progesterone from CL [Richards JS and Williams JJ, 1976]. However, the role of

prolactin in regulation of luteal function is not clear in cattle [Hansel W et al, 1973] and sheep [Niswender GD, 1974].

The action of LH on steroidogenic cells is mediated via cell membrane receptors. In cattle [Chegini N et al, 1991] and sheep [Harrison LM et al, 1987], the receptors for LH are present on both LLC and SLC, however only SLCs are responsive to progesterone stimulatory actions of LH. Upon binding to its receptors, LH activates adenylate cyclase to release the secondary messenger, cAMP. An enzyme, protein kinase A (PKA) is then activated by cAMP to mediate the actions of LH [Davis JS et al, 1996; Hoyer PB et al, 1984; Marsh JM, 1976]. It has been shown that LH does not affect the transcription or the activity of P-450_{scc} or 3β-HSD [Marsh JM, 1976; Wiltbank MC et al, 1993]. However, it seems that LH is required for the normal expression of StAR, P-450_{scc} and 3β-HSD during development of CL [Niswender GD et al, 2000]. The evidence has indicated that the acute stimulatory action of LH on progesterone secretion is independent of transcription mechanisms [Marsh JM, 1970]. It is now clear that PKA enhances progesterone synthesis by phosphorylation of StAR, thereby enhancing the rate of transport of cholesterol into the mitochondrial membrane [Arakane F et al, 1997; Epstein LF and Orme-Johnson NR, 1991]. In addition, LH has been shown to activate cholesterol esterase, however the amount of progesterone stimulation with this mechanism appears to be minimal [Wiltbank MC et al, 1993]. LH has been shown to activate phospholipase C/protein kinase C (PLC/PRKC) system in SLCs. However, the role this system in activation of progesterone is not clear [Davis JS et al, 1996]. Binding of LH to its receptors on LLC does not affect the intracellular concentrations of cAMP or increase the progesterone. However, LLC secrete large quantities of progesterone (> 80%) independent of LH stimulation and it appears that PKA is constitutively active in these cells [Hoyer PB et al, 1984]. In addition to LH, various other hormones influence the synthesis and secretion of progesterone from the luteal tissue. For example, growth hormone (GH) [Liebermann J and Schams D, 1994] and Insulin-like growth factor-1 (IGF-1) [Constantino CX et al, 1991; Devoto L et al, 1995] have been shown to increase the secretion of P4. Supporting their action, receptors for GH have been characterized in bovine, ovine, and rat luteal tissue [Carlsson B et al, 1993; Juengel JL et al, 1997; Lucy MC et al, 1993]. It has been shown that early CL produce greater amounts of prostaglandin E and I series, so authors suggested they have role in the development of CL. Accordingly, prostacyclin (PGI₂)

and PGE₂ stimulated progesterone synthesis from luteal tissues of cattle, sheep and humans [Alila HW et al, 1988a; Bennegard B et al, 1990; Fitz TA et al, 1984]. In addition, PGF_{2 α} stimulated luteal progesterone synthesis during early luteal phase [Choudhary E et al, 2005]. Ability of PGE₂ and PGF_{2 α} to stimulate progesterone synthesis has been shown to be important in preventing the apoptosis of steroidogenic cells [Bowolaksono A et al, 2008].

IV. Identification of PGF_{2 α} as a luteolytic factor

In general the estrous cycle consists of short follicular phase followed by long luteal phase. During the follicular phase, increasing concentration of estrogen secreted from growing follicles stimulates LH surge. This is followed by ovulation of the dominant follicle and formation of CL. If animal become pregnant, luteal function must be maintained throughout gestation, but if fertilization does not occur, luteal regression (luteolysis) has to occur for initiation of new ovarian cycle with another chance for pregnancy [McCracken JA et al, 1999]. The factors involved in regression of CL and their detailed cellular mechanism of action has been an area of intense investigation. During luteolysis two related events occur; first there is a loss or reduction in the capacity to synthesize and secrete progesterone. Subsequently, decline in progesterone is followed by the loss of luteal cellular components [Knickerbocker JJ et al, 1988; McGuire WJ et al, 1994]. Several observations have indicated that uterus is involved in the process of luteolysis. In 1923, Loeb was the first investigator to demonstrate that uterus might be involved in controlling the life span of CL by providing evidence that hysterectomy in guinea pigs extended life of the CL [Loeb L, 1923]. Subsequently, similar results were reported in other species such as pseudopregnant rats [Bradbury J, Brown WE, Gray LA, 1950], mice [Bartke A, 1970], hamsters [Caldwell BV et al, 1967], cattle, sheep [Wiltbank J, Casida, LE., 1956], and horses [Stabenfeldt GH et al, 1974]. In these animals, unilateral hysterectomy prolonged the life of the ipsilateral CL only if the vascular connection from the intact uterine horned is blocked [O'Shea JD et al, 1974]. However, hysterectomy did not prolong the length of the cycle or life of the CL in primates or humans, mouse, squirrel, opossum, and dogs [Niswender GD and Nett TM, 1994].

In 1969, Pharriss and Wyngarden [Pharriss BB and Wyngarden LJ, 1969] demonstrated that PGF_{2 α} brings about luteolysis in pseudopregnant rats. They reported that injections of large

amounts of $\text{PGF}_{2\alpha}$ (1mg/kg/day S.C) into rats shortened the length of pseudopregnancy and decreased the progesterone content of the CL. In sheep, intra-arterial infusion (carotid artery) of $\text{PGF}_{2\alpha}$ into the auto-transplanted ovary with vascular anastomoses to the vessels of the neck decreased the concentrations of progesterone in ovarian venous blood [McCracken J, 1971], which has been further elucidated [Bonnin P et al, 1999]. In sheep, it was demonstrated that $[\text{H}^3]\text{PGF}_{2\alpha}$ was transferred from uterine vein to ovarian artery by a countercurrent transfer mechanism [McCracken JA et al, 1971]. In addition, infusion of $[\text{H}^3]\text{PGF}_{2\alpha}$ into uterine vein appeared in adjacent ovary after a time period of 20-30 min [McCracken JA et al, 1972]. Similar experiments have demonstrated that $\text{PGF}_{2\alpha}$ is a luteolytic factor that is released from the uterus and delivered back to the corpus luteum, where it initiates the process of luteolysis. In cow, arachidonic acid released from endometrium was initially proposed to be a luteolytic substance [Hansel W et al, 1975]. Subsequently, $\text{PGF}_{2\alpha}$ has been shown to increase in an episodic manner in ovarian venous blood around the time of luteolysis [Nancarrow CD et al, 1973]. In agreement with this finding, PGFM, a metabolite of $\text{PGF}_{2\alpha}$ increased in peripheral blood of cattle during luteolysis [Kindahl H et al, 1980]. It is clear that $\text{PGF}_{2\alpha}$ is the primary luteolytic factor that initiates the process of luteolysis in most of the animal species studied for example pig [Bazer FW and Thatcher WW, 1977], mares [Douglas RH and Ginther OJ, 1976], goats [Homeida AM and Cooke RG, 1982], guinea pigs [Horton EW and Poyser NL, 1976], and rat [Pharriss BB and Wyngarden LJ, 1969].

V. $\text{PGF}_{2\alpha}$ biosynthesis and transport mechanism

Prostaglandins (PGs) belong to family of eicosanoids, which are unsaturated lipids derived from arachidonic acid ($\text{C}_{20:4, n-6}$) or similar polyunsaturated fatty acid precursors. Chemically, PGs are polyunsaturated 20-carbon fatty acids having a cyclopentane ring. PGs are designated by letters from A-J, indicating the nature and location of substitutes on the cyclopentane ring, and the position of double bonds within the ring. The numerical subscript (1, 2 or 3) has been attached based on the number of double bonds in the alkyl side chains. $\text{PGF}_{2\alpha}$ belongs to 2-series which are derived from eicosatetraenoic acid (arachidonic acid). The subscript alpha (α) indicates the spatial position of the hydroxyl group at C-9 in the cyclopentane ring and the molecule has additional double bonds at position 5 and 6 [Moore PK, 1985].

1. Biosynthesis of $\text{PGF}_{2\alpha}$ in Uterus

In uterus, $\text{PGF}_{2\alpha}$ is synthesized in endometrium and epithelial cells of endometrium synthesize large quantities of $\text{PGF}_{2\alpha}$, whereas, stromal cells preferentially synthesize PGE_2 [Fortier MA et al, 1988]. The release of arachidonic acid from membrane lipids is the first step in a series of enzymatic reactions leading to formation of $\text{PGF}_{2\alpha}$. These actions are mediated by a group of enzymes of the phospholipase A2 family [Clark JD et al, 1995]. These enzymes catalyze the release of fatty acids from sn-2 position of phospholipids. Two forms of PLA2 in mammalian cells are secretory PLA2 (sPLA2) and cytosolic PLA2 (cPLA2) [Ackermann EJ and Dennis EA, 1995; Clark JD et al, 1995; Dennis EA, 1994; Kudo I et al, 1993]. These enzymes are calcium (Ca^{2+})-independent and mediate the release of arachidonic acid in several cell types. cPLA2 preferentially catalyzes the release of arachidonic acid from sn-2 position of lipids [Clark JD et al, 1991; Sharp JD et al, 1991]. Intracellular Ca^{2+} is required for translocation and binding of cPLA2 to the membrane [Channon JY and Leslie CC, 1990]. cPLA2 can be activated by various cytokines and growth factors such as interleukin-1, tumor necrosis factor (TNF), colony-stimulating factor (CSF), epidermal growth factor, c-Kit ligand, and interferon- γ (IFN γ) [Clark JD et al, 1995]. The activity of cPLA2 has been localized primarily to nuclear membrane and endoplasmic reticulum (ER). Interestingly, these sites are shared by cyclooxygenase-2 (COX-2), an important enzymes involved in PG synthesis.

Next step during the synthesis of PG is conversion of arachidonic acid to an endoperoxide, PGH_2 , by prostaglandin G/H synthase (PGHS) (also called cyclooxygenase, COX). These enzymes are present on the luminal surface of ER and capable of moving between ER and nuclear membrane [Spencer AG et al, 1998]. Two biochemically identical forms of PGHS are PGHS-1 (COX-1) and PGHS-2 (COX-2), and share 60% of the sequence similarity [Smith WL et al, 1996]. Despite their similarities in the reaction catalyzed, they are subjected to different regulatory mechanism and have different mRNA stability [Dubois RN et al, 1998; Smith WL et al, 2000]. PGHS-1 is expressed constitutively in most of the mammalian tissues, whereas PGHS-2 is an inducible enzyme that can be induced rapidly by cytokines, growth factors and tumor inducers [Herschman HR, 1996]. PGH_2 can be utilized to make other types of prostanoids such as PGD_2 , PGE_2 , or $\text{PGF}_{2\alpha}$ by isomerase/reductases. PGH_2 can be directly converted into $\text{PGF}_{2\alpha}$ by 9,11-endoperoxide reductase commonly called as PGF synthase

(PGFS). It reduces the 9-, 11-endoperoxide group of PGH₂ to two -hydroxyl groups of PGF_{2α}. Alternatively, PGD₂ can be converted into PGF_{2α} by 11-ketoreductase or PGE₂ can be converted into PGF_{2α} by 9-keto-prostaglandin reductase (9K-PGR). In bovine endometrium, 9K-PGR has been shown to produce PGF_{2α} and it has an additional capacity to metabolize progesterone by its 20α-hydroxysteroid (20α-HSD) activity [Asselin E and Fortier MA, 2000]. In presence of NADPH, PGFS can convert PGD₂ to 9α, 11α -PGF₂, and these are involved in the contractibility of vascular smooth muscle and airway tract [Watanabe K, 2002].

In cow, three different types of PGFS have been isolated, the lung types PGFS1 [Watanabe K et al, 1985] and PGFS2 [Watanabe K et al, 1985], and the liver type dihydrodiol dehydrogenase 3 (DDBX) [Suzuki T et al, 1999]. PGFS purified from bovine lung is a 36.6 kDa monomeric protein and consists of 323 amino acids and it has high sequence similarity to family members of aldo-keto reductase (AKR). A recently identified AKR1B5 has been shown to be primarily responsible for the production of PGF_{2α} in bovine endometrium and it has capacity to metabolize progesterone due to its 20α-HSD activity [Madore E et al, 2003].

2. CL as an additional source of PGF_{2α}

In addition to uterus, corpora lutea of most mammalian species produce prostaglandins [Olofsson J and Leung PC, 1994] and CL is a rich source of arachidonic acid. Luteal cells from cattle [Milvae RA et al, 1983; Pate JL, 1988], sheep [Rexroad CE, Jr. and Guthrie HD, 1979], pseudopregnant rats [Olofsson J et al, 1992], pigs [Guthrie HD et al, 1978], and rhesus monkeys [Johnson MS et al, 1988] produce prostaglandins. Sheep CL express mRNAs for COX-1, COX-2, and prostaglandin metabolizing enzyme prostaglandin-15 dehydrogenase (PGDH) [Tsai SJ and Wiltbank MC, 1997]. Recently, CL of cattle have been shown to have machinery for biosynthesis and transport of PGs [Arosh JA et al, 2004]. These studies have indicated that bovine CL express constant amounts of COX-1, PGDH and PGFS (AKR1B5) throughout the lifespan of CL, whereas expression of PGES, PG transporter, receptor for PGE and PGF_{2α} vary with the stages of estrous cycle. Authors have suggested that CL preferentially produces PGF_{2α} during luteolysis, whereas greater amount of PGE₂ production occurs during the luteal maintenance.

3. Transport of $\text{PGF}_{2\alpha}$

$\text{PGF}_{2\alpha}$ synthesized in endometrium is transported to ovary by a utero-ovarian vascular pathway to initiate the process of luteolysis [McCracken JA et al, 1972]. The transfer of PGs from uterine venous vessels to ovarian artery primarily occurs at a specialized vascular network called utero-ovarian plexus (UOP) [Ginther OJ, 1981]. In pigs, the direction of release of endometrial $\text{PGF}_{2\alpha}$ differ depending on the need and function, for example it is preferentially released into circulation during luteolysis, whereas it is secreted directly into the lumen of uterus during maternal recognition of pregnancy [Bazer FW and Thatcher WW, 1977]. This directional transport of PGs cannot be explained by simple diffusion. PGs are charged anions and therefore have poor capacity to pass through biological membranes by simple diffusion and moreover the rate of transport of PGs by this mechanism is too slow to bring about their biological effects [Nelson DL and COX MM, 2000]. Therefore, it was proposed that the carrier-mediated proteins are required to selectively transport PGs. Prostaglandin transporter (PGT) has been identified in human liver [Lu R et al, 1996], rat kidney [Kanai N et al, 1995], and mouse lung [Pucci ML et al, 1999].

PGT is a polypeptide belonging to super family of 12-transmembrane organic anion transporting polypeptides (OATPs) [Schuster VL, 1998; Schuster VL, 2002]. It mediates efflux and influx of newly synthesized PGs and it is highly expressed in tissues producing large quantities of PGs [Bao Y et al, 2002]. In cattle, both mRNA and protein of PGT are expressed in endometrium, myometrium, and smooth muscle cells of UOP. Its expression pattern is consistent with its role in the compartmental transport of $\text{PGF}_{2\alpha}$ from uterus to ovary during luteolysis [Banu SK et al, 2003]. The compartmental transport of $\text{PGF}_{2\alpha}$ is important because of its high rate of clearance via lung, for example, 65% and 99% of PG is metabolized by single pass through the lung in cattle and sheep, respectively [McCracken JA et al, 1999]. Bovine CL expresses PGT, specifically on LLC and its mRNA expression is greatest during late luteal phase of the estrous cycle (Days 13-15). However the amount of protein remains constant throughout the estrous cycle. Therefore, authors suggested that PGT might play an important role in influx and efflux of available luteal PGE_2 or $\text{PGF}_{2\alpha}$ in a competitive manner for their autocrine or paracrine effects [Arosh JA et al, 2004].

VI. Regulation of PGF_{2α} synthesis and initiation of luteolysis;

The release of free AA from phospholipids is a rate-limiting step during PG synthesis [Kunze H and Vogt W, 1971; Lands WE and Samuelsson B, 1968]. Released AA is quickly converted to PGF_{2α} by cyclooxygenases [Vane JR and Botting RM, 1995]. However, during luteolysis, PGF_{2α} is secreted from uterus in the form of 4 to 8 discrete pulses at intervals of 6 to 8 h [Nancarrow CD et al, 1973; Thorburn GD et al, 1973]. The exact mechanism of the initiation of PGF_{2α} synthesis from uterus is not clearly understood. In sheep, pulses of oxytocin or neurophysin occurred concurrently with the PGFM pulses during luteolysis [Hooper SB et al, 1986]. In agreement, large pulses of oxytocin/neurophysin have been reported to occur in cattle [Walters DL and Schallenberger E, 1984] and goats [Cooke RG and Homeida AM, 1984] during luteolysis. In addition, oxytocin stimulated the synthesis of PGF_{2α} in the uterus [Roberts JS and McCracken JA, 1976; Sharma SC and Fitzpatrick RJ, 1974][Schams D, 1989]. Neurophysin is released in an episodic manner lasting for few minutes at a frequency of 3 pulses per hour in sheep during follicular phase and in ovariectomized ewes during estradiol replacement [McCracken JA et al, 1991]. Authors of this study indicated that an episodic pattern of release of oxytocin might cause large pulsatile episodes of uterine PGF_{2α}, proposing that neurohypophyseal oxytocin might act as a central pulse generator signal.

In cattle and sheep, corpora lutea secrete large quantities of oxytocin [Fields PA et al, 1983][Wathes DC and Swann RW, 1982]. In cattle and sheep, LLC appear to be the cellular source of oxytocin [Fields MJ and Fields PA, 1986; Fields PA et al, 1983][Rodgers RJ et al, 1983]. Moreover, in cattle exogenous PGF_{2α} increased oxytocin in jugular venous blood that peaked within 15-20 min [Schams D and Karg H, 1982]. In sheep, treatment with cloprostenol, an analog of PGF_{2α}, increased the secretion of oxytocin from the ovary with the CL and not from the opposite ovary or the brain [Flint AP and Sheldrick EL, 1982]. This study clearly indicated that the oxytocin came from the CL and not from neurohypophysis following PGF_{2α} injection. It was proposed that oxytocin synthesized in CL is totally discharged with each pulse of PGF_{2α}, and an interval between pulses of PGF_{2α} might be required to re-synthesize luteal oxytocin for subsequent secretion [Flint AP et al, 1990]. However, this was shown to be unlikely because of the low mRNA for oxytocin during luteolysis in sheep [Ivell R et al, 1990]. The contribution of neurohypophysis to circulating oxytocin during luteolysis is ~10%, whereas

~50% from CL. However, as luteolysis progresses, the relative contribution of oxytocin from the neurophysis is proportionately greater [McCracken JA et al, 1996]. Regardless of the magnitudes of contributions of oxytocin from CL or neurohypophysis, it has been proposed that small increases in circulating concentrations of oxytocin, due to central oxytocin pulse generator, stimulate the subluteolytic amounts of $\text{PGF}_{2\alpha}$, from uterus, which in turn stimulate large supplemental release of luteal oxytocin [McCracken JA et al, 1999]. Subsequently, stimulating large quantities of uterine $\text{PGF}_{2\alpha}$, to initiate the process of luteolysis. Importantly, in sheep both *in vivo* and *in vitro* treatment with $\text{PGF}_{2\alpha}$, stimulated the luteal production of $\text{PGF}_{2\alpha}$ [Tsai SJ and Wiltbank MC, 1997]. The authors of these studies have proposed that local production of luteal $\text{PGF}_{2\alpha}$ can act in an autocrine or paracrine manner to increase the luteolytic effect of uterine $\text{PGF}_{2\alpha}$, by forming positive feedback loop during luteolysis.

It has been shown that the ability of oxytocin to stimulate uterine $\text{PGF}_{2\alpha}$, synthesis in ovariectomized animals depends on the pre-exposure to progesterone and can be enhanced by acute or chronic treatment with estrogen. In ovariectomized cows and ewes, oxytocin stimulated the secretion of uterine $\text{PGF}_{2\alpha}$, only after animals had been exposed to progesterone for 7-10 days [Homanics GE and Silvia WJ, 1988; Lafrance M and Goff AK, 1988]. The pre-exposure of uterus to progesterone has been proposed to prime the uterus for the release of $\text{PGF}_{2\alpha}$ by increasing the accumulation of lipid precursors [Nissenson R et al, 1978; Soloff MS et al, 1983] [Boshier DP and Holloway H, 1973; McCracken JA, 1980]. It has been shown that progesterone prevented the ability of estrogen to up-regulate oxytocin receptors [Leavitt WW et al, 1985]. In addition, progesterone inhibited the action of oxytocin in uterus by non-genomic action by changing the conformation of oxytocin receptors [Grazzini E et al, 1998]. In sheep, withdrawal of progesterone increased the receptors for oxytocin in endometrium within 6 h. Importantly, exposure to progesterone for 7-14 days down-regulated its own receptor in endometrium [Clarke CL, 1990; Milgrom E et al, 1973] and hypothalamus [Blaustein JD and Feder HH, 1979; Moguilewsky M and Raynaud JP, 1979]. Therefore, a model has been proposed for hormonal regulation of $\text{PGF}_{2\alpha}$ and initiation of luteolysis as follows (reviewed by [McCracken JA et al, 1999]). 1) The loss of progesterone receptors during the late luteal phase prevents the suppressing effect of progesterone on oxytocin receptors and allows estrogen to upregulate estrogen receptors in uterus, 2) Returning action of estrogen will stimulate the release of hypothalamic oxytocin pulse generator to stimulate low episodic levels of oxytocin, 3) Oxytocin

will then stimulate the subluteolytic release of $\text{PGF}_{2\alpha}$ from uterus, which in turn stimulates the additional release of oxytocin from CL, 4) Increased oxytocin will further stimulate the secretion of $\text{PGF}_{2\alpha}$ from uterus and CL in positive feedback manner, 5) Additionally, $\text{PGF}_{2\alpha}$ released from CL stimulate its own synthesis of $\text{PGF}_{2\alpha}$ in a autocrine manner to complete the process of luteolysis.

VII. $\text{PGF}_{2\alpha}$ signaling during luteolysis:

1. Receptors for $\text{PGF}_{2\alpha}$

Fried et al. [Fried J et al, 1969] were the first to provide evidence for presence of $\text{PGF}_{2\alpha}$ receptors in endometrium by showing that 7-oxa-acetylenic analogs of $\text{PGF}_{1\alpha}$ prevented the contractibility of uterus. Later, binding sites for $\text{PGF}_{2\alpha}$ in CL have been identified in sheep [Balapure AK et al, 1989; Powell WS et al, 1974], rat [Busmann LE, 1989], pig [Gadsby JE et al, 1990], and humans [Rao CV et al, 1977]. Subsequently, receptors for $\text{PGF}_{2\alpha}$ (FP) have been cloned and characterized in various tissues including CL in cattle [Sakamoto K et al, 1994], human [Lake S et al, 1994], rat [Kitanaka J et al, 1994], mouse [Sugimoto Y et al, 1994], and sheep [Graves PE et al, 1995]. FP receptors are members of seven-transmembrane domain receptor family and coupled to G-protein. It appears that in cattle there is single gene for FP consisting of 40 kilobases (kb)[Ezashi T et al, 1997] and organized with three exons and two introns, which is conserved across human, mouse, and cattle [Betz R et al, 1999; Hasumoto K et al, 1997]. The molecular weight of FP is 40 kDa and it appears to be similar among species. The open reading frame of FP consists of 362 amino acid residues in cattle and sheep, 366 in mouse and rat, and 359 amino acids in human beings. The homology of bovine FP amino acid sequence is 98% with ovine, 86% with human beings, 80% with murine and 78% with rat [Anderson LE et al, 2001]. There are two isoforms of FP, FP_A and FP_B [Pierce KL et al, 1997]. These isoforms have been suggested to arise from alternative mRNA splicing mechanism. FP_B is a truncated form of FP_A , lacking 46 amino acids at the carboxy-terminal end and four putative protein kinase C (PKC) phosphorylation sites. FP_A isoform is phosphorylated by $\text{PGF}_{2\alpha}$ via PKC dependent pathway, whereas FP_B is not phosphorylated, so it was suggested that FP isoforms are regulated differentially by $\text{PGF}_{2\alpha}$ [Fujino H et al, 2000]. In cattle, mRNAs for FP receptor are expressed on LLC, SLC, and endothelial cells [Mamluk R et al, 1998], whereas in

sheep, they are present on only LLC [Juengel JL et al, 1996] and expression of FP on luteal endothelial cells has not been confirmed. In cattle, FP mRNAs increased from early to late phase of estrous cycle, and decreased markedly in regressing CL [Sakamoto K et al, 1995]. Similar pattern of expression was reported in sheep [Graves PE et al, 1995] and pig [Gadsby JE et al, 1990] CL. Importantly, in cattle and sheep, *in vivo* and *in vitro* treatments with PGF_{2α} decreased the FP receptors similar to that observed during natural luteolysis [Juengel JL et al, 2000; Mamluk R et al, 1998]. The physiological significance of down-regulation of FP receptors during luteolysis is not clear,

2a. PRKC/calcium signaling pathway

PGF_{2α} has been shown to affect composition and fluidity of the luteal lipid membrane [Carlson JC et al, 1984; Leung PC et al, 1986; Raymond V et al, 1983]. Later it was shown that the action of PGF_{2α} might be mediated through an increase in intracellular Ca²⁺ derived from internal sources in rat luteal cells [Dorflinger LJ et al, 1984]. Around the same time, it was reported that hydrolysis of phosphatidylinositol 4, 5 –biphosphate by phospholipase C (PLC) leads to the generation of secondary messenger inositol 1-4-5-trisphosphate (IP₃), which in turn stimulated the release of Ca²⁺ from ER [Berridge MJ and Irvine RF, 1984; Spat A et al, 1986]. Similarly, in steroidogenic cells of the ruminant CL, PGF_{2α} activated G-protein coupled FP receptors leading to activation of PLC. Once active, PLC acts on membrane lipids to produce intracellular secondary messengers such as IP₃ and diacylglycerol (DAG) [Davis JS et al, 1988]. Accordingly, in bovine luteal cells, hydrolysis of phosphatidylinositol 4, 5 –biphosphate and mobilization of intracellular Ca²⁺ were stimulated by PGF_{2α} [Davis JS et al, 1987b]. Furthermore, Ca²⁺ and PRKC mediated the intracellular actions of PGF_{2α} in luteal cells [Wiltbank MC et al, 1991]. In bovine luteal cells, PGF_{2α} activated the Raf/MEK1/mitogen-activated protein kinase (MAPK) signaling pathway [Chen DB et al, 1998]. Activation of this pathway by PGF_{2α} increased the expression of c-fos and c-jun and activated transcription factors called activator protein-1 (AP-1). More recently, it has been shown that activation of Raf/MEK1/MAPK pathway by PGF_{2α} is mediated by PRKC in bovine luteal cells [Chen D et al, 2001] [Stocco C et al, 2007]. The constituents of AP-1 transcription factors, *c-fos* and *c-jun*, regulated the expression of genes having AP-1 binding site on their promotor. Therefore, it was proposed that activation of AP-1 by PGF_{2α} in bovine luteal cells might modulate the expression

of genes during luteolysis. However, the detailed cellular and molecular mechanisms of luteolysis initiated by $\text{PGF}_{2\alpha}$ are not understood. The schematic representation of $\text{PGF}_{2\alpha}$ signaling pathway in bovine CL is indicated in figure 1.

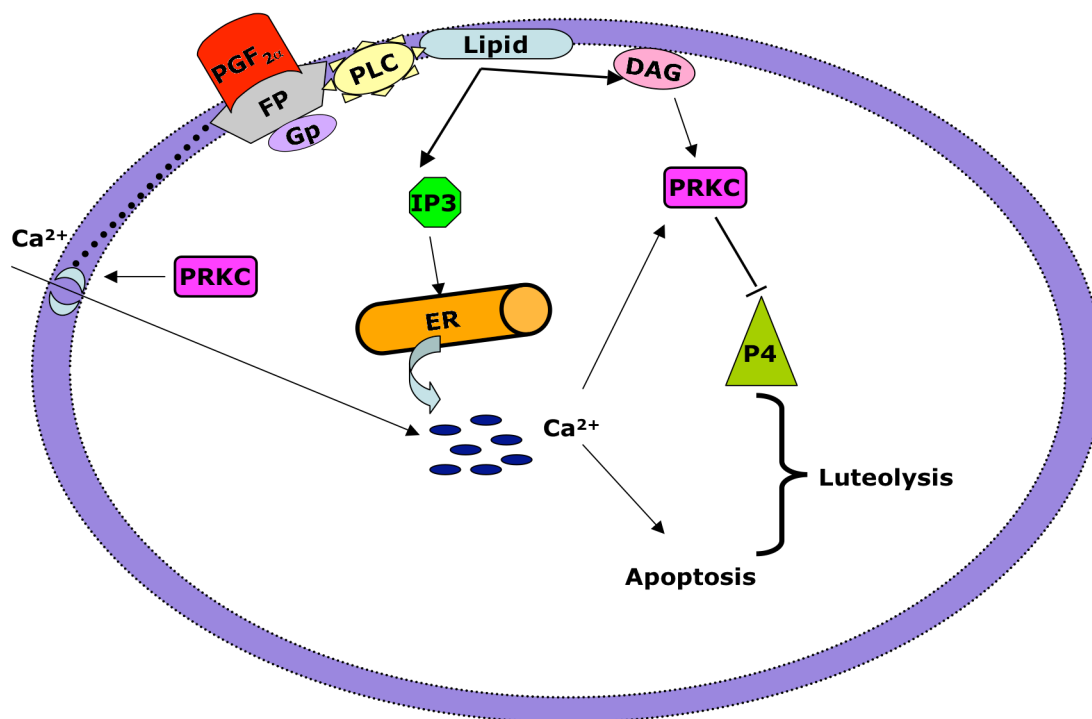


Figure 1. $\text{PGF}_{2\alpha}$ signaling pathway: PLC: phospholipase C; $\text{PGF}_{2\alpha}$: $\text{PGF}_{2\alpha}$ receptor; ER: endoplasmic reticulum; PRKC: protein kinase C; P4: progesterone; Gp: G-protein; DAG: diacylglycerol; IP3: inositoltriphosphate

2b. PRKC

Protein kinase C (PRKC) is a family of serine/threonine kinases with 11 isozymes. These enzymes are single polypeptide chain with regulatory region located at amino-terminal region having molecular weight of 20-70 kDa, whereas the catalytic unit is located at the carboxy-terminal end with molecular weight of 45 kDa. Different isozymes have different subcellular localization and co-factor requirements [Nishizuka Y, 1988; Quest AF, 1996]. The conventional PRKC category includes four isozymes, alpha (α), beta I (β I), beta II (β II), and gamma (γ). The

novel PRKC category includes four additional isozymes, delta (δ), epsilon (ϵ), theta (θ), and eta (η). Finally, three more isozymes make up the atypical PRKC group, lambda (λ), zeta (ζ), and mu (μ). The conventional isozymes are activated by diacylglycerol (DAG), Ca^{2+} , and

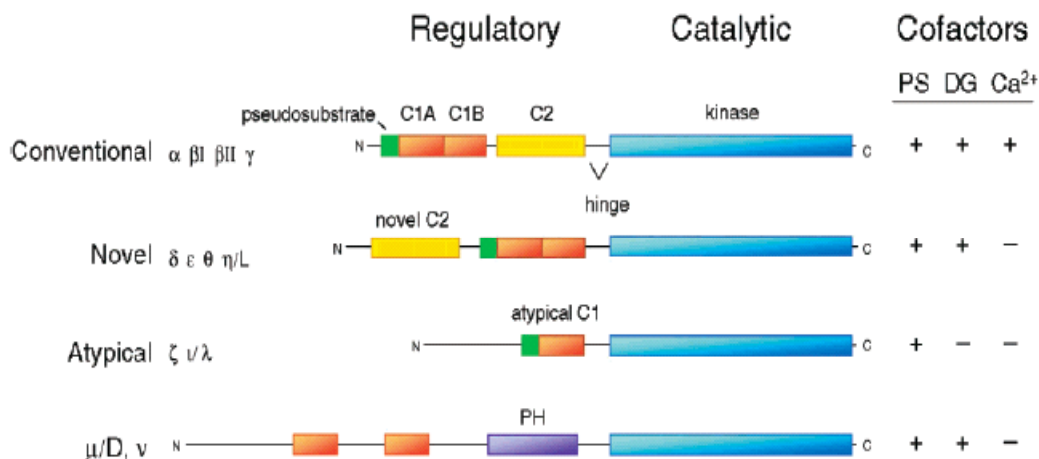


Figure 2. PRKC isozyme specific domains and co-factor requirement (adapted from [Newton AC, 2001]).

phosphatidylserine, whereas, the novel isozymes depend on DAG and phosphatidylserine for their activation and atypical isozymes are activated by phosphatidylserine. The regulatory unit consists of two important domains, one is an autoinhibitory sequence (pseudosubstrate) and the other includes one or two membrane targeting modules (C1 and C2). Pseudosubstrate allosterically regulates the enzyme activity. During the inactive state of an enzyme, pseudosubstrate covers the substrate-binding site. Whenever the enzyme is activated by co-factor or co-factor-independent mechanisms, there will be a release of pseudosubstrate from the kinase core [Orr JW and Newton AC, 1994b; Orr JW and Newton AC, 1994a]. It has been shown that increased intracellular Ca^{2+} is essential for translocation of PRKC to membrane by increasing its affinity towards anionic lipids. Ca^{2+} binding with the enzyme engages C2 domain with the lipid membrane, thereby decreasing the dimensionality and increasing the probability of engaging C1 domain with DAG. Binding of PRKC domains to membrane releases the energy required to release pseudosubstrate from an active catalytic site, thereby activating the enzyme [Johnson JE et al, 2000]. In addition DAG, and phorbol esters act by increasing the affinity of PRKC to membranes by acting as molecular glue. It has been shown that the DAG binding to C1 domain results in presentation of contiguous hydrophobic surface, allowing increased

affinity towards lipid membrane [Zhang G et al, 1995]. However, the action of DAG does not last long because of its rapid metabolism and therefore phorbol esters are 2 times more potent than DAG. In addition to these regulatory molecules, the anchoring proteins of PRKC are very important for translocation to its substrate, or regulators such as phosphatases and kinases, or to specific intracellular compartments [Mochly-Rosen D, 1995]. Accordingly, peptide agonists can be used to activate PRKC enzymes that are regulated by the anchoring proteins such as receptors for activated kinases (RACKs) [Csukai M and Mochly-Rosen D, 1999]. It has been proposed that the site on the enzymes that binds to anchoring proteins are masked by masking domain called pseudo-RACK sequence. This sequence is similar to the sequence of binding site on the anchoring protein. Therefore, peptide agonists similar to this sequence can disrupt intramolecular interaction by disengaging the pseudosubstrate RACK sequence from anchoring to the protein-binding site, thereby activating the enzyme. Accordingly, it has been shown that C2 domain of PKC ϵ (official symbol, PRKCE) has a pseudo-RACK sequence NDAPIGYD (V1 region) [Csukai M et al, 1997; Dorn GW, 2nd et al, 1999]. Agonists or antagonist with sequences similar to pseudo-RACK has been extensively utilized to activate or inactivate PRKCE in several cell systems.

Pharmacological activation of PKC with the phorbol esters inhibited steroidogenesis in luteal cells from human [Abayasekara DR et al, 1993] and sheep [Wiltbank MC et al, 1991]. In sheep luteal cells, PGF $_{2\alpha}$ prevented the lipoprotein-stimulated progesterone synthesis and had no effect in PRKC-deficient cells [Wiltbank MC et al, 1990]. It has been shown that anti-steroidogenic actions of PRKC might be due its ability to inhibit the expression of mRNAs encoding P-450 $_{scc}$ and 3 β -HSD [McGuire WJ et al, 1994]. Moreover, in rat testicular microsomes, the activation of PLC activated PRKC resulting in decreased activity of 3 β -HSD, which suggested that PRKC might modulate the activity of 3 β -HSD [Cooke GM and Robaire B, 1988]. In addition, activation of PRKC inhibited the transport of cholesterol across mitochondrial membrane [Wiltbank MC et al, 1993]. However, the detailed cellular mechanism by which PRKCs mediate the anti-steroidogenic actions of PGF $_{2\alpha}$ needs further investigation.

2c. Ca²⁺-calmodulin dependent kinase kinase 2, beta (official symbol, CAMKK2/CAMKK β)

CAMKK is a recently discovered enzyme and first discovered in rat brain, where it activated an inactive recombinant CaMK1V [Okuno S and Fujisawa H, 1993]. CAMKKs belong to protein kinase superfamily and perform the function of serine/threonine phosphorylation. Two types of CAMKK have been characterized in rat brain, CAMKK alpha and CAMKK beta [Edelman AM et al, 1996]. Bovine CAMKK2 is a 63 kDa protein and consists of 579 amino acids. Both forms are expressed from distinct genes and both appear to be activated by Ca²⁺-CaM and capable of Ca²⁺-CaM-dependent autophosphorylation. Both forms activated downstream enzymes, CaMK1 and CaMK1V by phosphorylation-dependent mechanism. Accordingly, CAMMK2 stimulated CaMK1 by 25-fold and CaMKIV by 12-fold by phosphorylating amino acid residues at Thr¹⁷⁷ and Thr²⁰⁰, respectively. However, in brain CAMKK2 and CaMK1V were co-localized in cerebellar region suggesting that CaMK1V might be acutely regulated by CAMKK2 [Anderson KA et al, 1998]. It has been suggested that CAMKK alpha might specifically regulate CaMK1. However, because of its ubiquitous distribution, it is unclear which of the isoforms regulate the activity of CaMK1 [Haribabu B et al, 1995; Picciotto MR et al, 1993]. Similar to other CaM kinase family, CAMKK2 has capacity to undergo an autophosphorylation upon binding with Ca²⁺-CaM. Phosphorylation will prevent the reformation of autoinhibitory conformation, thereby keeping the enzyme in an autonomously active state [Braun AP and Schulman H, 1995]. This property of autophosphorylation is observed with CaMK1 and CaMK1V. The activity of CAMKK2 is independent of Ca²⁺-CaM binding and subsequent autophosphorylation, however, these mechanisms can enhance its activity [Edelman AM et al, 1996]. Nevertheless, it has been proposed that these mechanisms *in vivo* might have physiological consequences such as cellular distribution and protein-protein interaction with downstream signaling molecules. Neuronal tissue has been shown to be primary site of expression and expressed at lower amounts in testis, thymus, and spleen [Anderson KA et al, 1998; Tokumitsu H et al, 1995]. CaMK IV is expressed in steroidogenic tissues such as ovary, testis, and adrenal gland in addition to brain, thymus, and bone marrow, whereas, CaMKI is ubiquitously expressed [Haribabu B et al, 1995; Means AR et al, 1997]. However, there is no evidence on the expression and role of CAMKK2 in ovarian tissue to date. In rabbit aortic smooth muscle cells, epinephrine stimulated the activity of CaMKII followed by increase in mobilization of

arachidonic acid [Muthalif MM et al, 1996]. In addition, Ca^{2+} play a very important role in oxytocin-induced $\text{PGF}_{2\alpha}$ release in bovine endometrium [Burns PD et al, 1998]. In agreement, oxytocin stimulated $\text{PGF}_{2\alpha}$ synthesis in ovine endometrium by activating ERK1/2. The activation of ERK1/2 depends on Ca^{2+} -CaM in luteinized granulosa cells [Stocco CO et al, 2002]. Therefore, CAMKK2 might play a very important role in stimulation of $\text{PGF}_{2\alpha}$ synthesis from uterus and CL. In addition, the possibility of direct participation of CAMKK2 in mediating the antisteroidogenic actions of the rise in intracellular Ca^{2+} stimulated by $\text{PGF}_{2\alpha}$ needs further investigation.

VIII. Functional luteolysis: inhibition of progesterone synthesis

The pulsatile release of $\text{PGF}_{2\alpha}$ from the uterus around 17-18 days of estrous cycle initiates the process of luteolysis in the ruminant CL [Kindahl H et al, 1976; Wolfenson D et al, 1985] [Shirasuna K et al, 2004]. In cattle, direct measurement of $\text{PGF}_{2\alpha}$ in uterine-venous blood indicated that concentrations of $\text{PGF}_{2\alpha}$ increase on Day 14 and remain elevated on Days 15-20 [Shemesh M and Hansel W, 1975b]. It has been suggested that finite number of frequent $\text{PGF}_{2\alpha}$ pulses occurring over a period of ~24 h was necessary for the initiation of functional luteolysis. Accordingly, $\text{PGF}_{2\alpha}$ inhibited the synthesis of progesterone *in vivo* in cattle, sheep, pigs, monkeys, human beings, pseudopregnant rats and rabbits [Niswender GD and Nett TM, 1994]. Similarly, *in vitro* $\text{PGF}_{2\alpha}$ treatment inhibited the synthesis and secretion of progesterone in luteal cells of mid to late CL [Niswender GD et al, 2000]. The decline in progesterone occurs over a period of ~ 24-36 h and reduction in progesterone starts after the first pulse of $\text{PGF}_{2\alpha}$ in sheep, whereas in monkeys, the decline in progesterone occur over a period of ~48 h.

1. Antisteroidogenic actions of $\text{PGF}_{2\alpha}$

Juengel et al [Juengel JL et al, 1994] suggested that the down-regulation of receptors for LH by $\text{PGF}_{2\alpha}$ might be one of the mechanisms for antisteroidogenic actions of $\text{PGF}_{2\alpha}$. However, in cows [Spicer LJ et al, 1981] and ewes [Diekman MA et al, 1978], the decline in progesterone preceded the decrease in mRNA for LH receptor. It had been shown that $\text{PGF}_{2\alpha}$ might interfere with progesterone stimulatory actions of LH by affecting PKA. Accordingly, it was reported that the activity of PKA might be reduced by an increased degradation of cAMP due to the

activation of phosphodiesterase enzyme [Agudo LS et al, 1984; Garverick HA et al, 1985]. This mechanism might be important in inhibiting the expression of StAR, because cAMP activated the promoter for StAR in MA-10 cells. LLC account for the most of the basal progesterone due to constitutively high activity of PKA [Diaz FJ et al, 2002]. Therefore, decrease in the activity of PKA by $\text{PGF}_{2\alpha}$ might be responsible for the inhibition of progesterone synthesis in LLC. $\text{PGF}_{2\alpha}$ decreased sterol carrier protein (SCP-2) [McLean MP et al, 1995] and cytoskeleton [Murdoch WJ, 1996] that are involved in cholesterol transport. Negative regulation of progesterone synthesis by affecting the activity of StAR has been suggested to be the major point of regulation by $\text{PGF}_{2\alpha}$. Both, *in vivo* and *in vitro* treatment of luteal tissues with $\text{PGF}_{2\alpha}$ decreased the expression of StAR mRNA in sheep and cattle. In addition, it was proposed that that $\text{PGF}_{2\alpha}$ might inhibit the translation of StAR mRNA. The orphan nuclear receptor, DAX-1 was proposed to mediate the inhibitory action of $\text{PGF}_{2\alpha}$ on StAR mRNA expression, because DAX-1 bound to a DNA hairpin structure on the StAR promoter. Accordingly, $\text{PGF}_{2\alpha}$ induced DAX-1 RNA and inhibited progesterone synthesis in rat CL [Sandhoff TW and McLean MP, 1999; Zazopoulos E et al, 1997]. Activation of the proteasome system by $\text{PGF}_{2\alpha}$ might be another mechanism of regulation of StAR, because the inhibitors of proteasome system increased StAR protein and stimulated progesterone synthesis in rat and human granulosa-luteal cells [Tajima K et al, 2001]. Phosphorylation of StAR at positions Ser194/195 by PKA increased the steroidogenic activity [Arakane F et al, 1997]. Therefore, inhibitory actions of $\text{PGF}_{2\alpha}$ on PKA activity might reduce the ability of StAR to transport cholesterol into mitochondria. Activation of PKC partially inhibited progesterone in luteal cells supplied with 25-hydroxycholesterol and this effect was abolished in PKC deficient cells, which suggested that PKC inhibited cholesterol side chain cleavage enzyme [Wiltbank MC et al, 1990]. However, inhibition of progesterone by $\text{PGF}_{2\alpha}$ was not associated with decrease in mRNA or protein of P450Scc enzyme complex [Belfiore CJ et al, 1994; Rodgers RJ et al, 1995]. In addition, $\text{PGF}_{2\alpha}$ did not reduced 3β -HSD during its initial 24 h of treatment. Therefore, it was proposed that progesterone inhibitory actions of $\text{PGF}_{2\alpha}$ might not be mediated by inhibition of P450Scc or 3β -HSD.

In cattle, FP receptors are present on both LLC and SLC, however in sheep high affinity FP receptors are absent on SLC [Fitz TA et al, 1982] [Wiltbank MC et al, 1993]. However,

activation of PRKC inhibited PKA-stimulated progesterone synthesis in SLC suggesting that antisteroidogenic actions of $\text{PGF}_{2\alpha}$ in ovine SLC might be indirect. Therefore, the identity of the factor that activate PKC in ovine CL was not known until recent. The receptors for oxytocin are present on SLC and treatment of luteal tissues with oxytocin decreased the secretion of progesterone [Bennegard-Eden B et al, 1995; Pitzel L et al, 1993]. In addition, $\text{PGF}_{2\alpha}$ stimulated the secretion of oxytocin from luteal tissue [Flint AP and Sheldrick EL, 1982]. Therefore, it is tempting to speculate that oxytocin might be the ideal candidate to mediate antisteroidogenic actions of $\text{PGF}_{2\alpha}$ in SLC. More recently, it was shown that oxytocin stimulated increase in intracellular Ca^{2+} in SLC, and this action was abolished by progesterone [Niswender GD et al, 2007]. These authors have proposed that $\text{PGF}_{2\alpha}$ stimulate LLC to secrete oxytocin, which then bind to its receptors on SLC and inhibit synthesis of progesterone. Once the intraluteal concentrations of progesterone declines, then oxytocin might induce the apoptosis of SLC by raising $[\text{Ca}^{2+}]_i$.

2. Mediators of antisteroidogenic actions of $\text{PGF}_{2\alpha}$

In cattle, $\text{PGF}_{2\alpha}$ increased endothelin-1 (EDN1) in regressing CL and ovarian venous blood, which suggested that EDN1 might be a mediator of luteolysis [Ohtani M et al, 1998]. In addition, EDN1 and angiotensin II (AngII) inhibited progesterone synthesis in bovine luteal cells [Girsh E et al, 1996; Miyamoto A et al, 1997]. Both LLC and SLC express mRNA for ET type A receptor (ETA) [Meidan R et al, 1999] and action of EDN1 is mediated through selective ETA type receptor [Girsh E et al, 1996]. Therefore, it is clear that in cattle, EDN1 mediate the antisteroidogenic actions of $\text{PGF}_{2\alpha}$. Delivery of EDNRA and EDNRB receptor antagonists into the ovine CL inhibited progesterone inhibitory actions of $\text{PGF}_{2\alpha}$ during first 12 h, which indicated the role of EDN1 in mediating the antisteroidogenic actions of $\text{PGF}_{2\alpha}$ [Doerr MD et al, 2008]. In cattle, a donor of nitric oxide (NO) inhibited the synthesis of progesterone in luteal cells [Skarzynski DJ and Okuda K, 2000]. In addition, administration of NO synthase (NOS) inhibitor to cow inhibited luteolytic actions of $\text{PGF}_{2\alpha}$, which was indicated by prolonged length of estrous cycle [Skarzynski DJ et al, 2003]. NO mediated the acute increase in luteal blood flow during first 30 min to 2 h after $\text{PGF}_{2\alpha}$ administration. Accordingly, in cattle, $\text{PGF}_{2\alpha}$ - induced expression of endothelial nitric oxide synthase (eNOS) in the periphery of CL and NO

donor induced acute increase in the luteal blood flow and decreased the length of estrous cycle [Acosta TJ et al, 2002; Shirasuna K et al, 2008]. This acute increase in blood flow has been proposed to be important in stimulating capillary endothelial cells to secrete vasoactive amines such as EDN1 and Ang II. These vasoactive amines facilitate a decrease in the luteal blood flow by vasoconstriction and inhibition of progesterone synthesis [Acosta TJ et al, 2002; Ohtani M et al, 1998]. In addition, early luteal resistance to $\text{PGF}_{2\alpha}$ was suggested to be due to the lack of ability of $\text{PGF}_{2\alpha}$ to induce eNOS in the periphery of CL and subsequent absence of acute increase in luteal blood flow [Shirasuna K et al, 2008].

However, it has been shown that there is an inverse relationship between the expression of EDN1 and NOS throughout the estrous cycle. The expression of NOS (both eNOS and iNOS) was elevated in the early CL and declined towards the end of cycle, whereas the expression of EDN1 increased during luteolysis. In addition, NO inhibited the expression of EDN1 in luteal endothelial cells. Therefore, it was proposed that low amounts of NO during luteolysis might facilitate an increased expression of EDN1 and greater amounts of NO during the early luteal stages might be responsible for low EDN1 expression [Rosiansky-Sultan M et al, 2006]. In addition, lower expression of EDN1 during early CL and inability of $\text{PGF}_{2\alpha}$ to induce its expression has been suggested to be part of mechanism responsible for early luteal resistance to $\text{PGF}_{2\alpha}$. However, the expression pattern of NOS and its role throughout the estrous cycle remains controversial.

In cattle, tumor necrosis factor alpha ($\text{TNF}\alpha$) and its receptors are expressed in CL [Sakumoto R et al, 2000]. In cattle and sheep, $\text{TNF}\alpha$ secretion was increased in regressing CL suggesting its role during luteolysis [Ji I et al, 1991; Shaw DW and Britt JH, 1995]. In cattle, $\text{TNF}\alpha$ inhibited *in vivo* progesterone synthesis in CL only after pre-exposure to $\text{PGF}_{2\alpha}$ and EDN1 [Ohtani M et al, 2004]. Supporting this observation, $\text{TNF}\alpha$ and $\text{PGF}_{2\alpha}$ were synergistically inhibited progesterone synthesis in porcine CL [Wuttke W et al, 1998]. Therefore, it was proposed that $\text{TNF}\alpha$ facilitates a rapid decline in progesterone synthesis after initial decline by $\text{PGF}_{2\alpha}$ and EDN1.

IX. Structural regression of CL

Structural regression is characterized by decrease in the weight and size of CL. In cattle, structural luteolysis started 12h after an initial functional luteolysis following the administration

of $\text{PGF}_{2\alpha}$ [Neuvians TP et al, 2004]. As a consequence, the size of the regressed CL is greatly reduced to a tiny white scary structure called corpus albicans, which is eventually reabsorbed and replaced with ovarian stroma. It is well established that the structural regression of CL occurs by the process of apoptosis of luteal and endothelial cells. Morphological changes were not evident until 24-36 h after $\text{PGF}_{2\alpha}$ injection and interestingly endothelial cells were first to undergo apoptosis [Sawyer HR et al, 1990]. There is a substantial amount of evidence supporting the participation of immune cells in structural luteolysis [Murdoch WJ et al, 1988]. Injection of $\text{PGF}_{2\alpha}$ into mid cycle cows and ewes resulted in an increased expression of monocyte chemo-attractant protein-1 (MCP-1), which is a potent chemo-attractant to immune cells [Tsai SJ et al, 1997]. In addition, MCP-1 was highly expressed in regressing rat CL [Townson DH et al, 1996]. Therefore, MCP-1 appears to be initial trigger for the infiltration of immune cells during luteolysis. The primary role of macrophages during luteolysis appears to be phagocytosis of apoptotic luteal cells and degradation of the extracellular matrix [Paavola LG, 1979; Pepperell JR et al, 1992]. In addition, T-lymphocytes secrete interferon- γ (IFN- γ) that induces the expression of major histocompatibility complex antigens on cultured bovine luteal cells [Fairchild DL and Pate JL, 1989]. The cytokines, $\text{TNF}\alpha$, IL-1, and IFN- γ were synergistically stimulated the synthesis of $\text{PGF}_{2\alpha}$ from bovine luteal cells [Benyo DF and Pate JL, 1992; Nothnick WB and Pate JL, 1990]. These cytokines induced apoptosis in various cell types [Gupta S, 2003]. In cattle, Fas and Fas-ligand system appear to play an important role in the regulation of luteal cell- apoptosis. For example, expression of Fas is greater during structural regression of CL (Days 19-21). Furthermore, IFN- γ increased the expression of Fas mRNA in luteal cells. Importantly, Fas-ligand induced apoptosis in luteal cells that were pre-exposed to IFN- γ alone or with $\text{TNF}\alpha$ [Taniguchi H et al, 2002]. In addition, progesterone antagonist increased the expression of Fas mRNA and subsequent treatment with Fas ligand induced an apoptosis in bovine luteal cells [Okuda K et al, 2004]. This observation strongly supports the luteo-protective role of progesterone and it could be the reason for initiation of structural luteolysis after decline in progesterone.

$\text{PGF}_{2\alpha}$ induced apoptosis in luteal cells of several species [Niswender GD et al, 2000]. Formation of DNA ladder is an indication of cells undergoing apoptosis. In cattle, administration of $\text{PGF}_{2\alpha}$ induced DNA ladder formation in CL after 24-48 h [Juengel JL et al,

1993]. The pro-apoptotic protein mRNAs of bax and caspase-3 were increased in regressing CL on Days 21 of cycle, whereas low amounts of these mRNAs were observed in pregnant CL [Rueda BR et al, 1997]. In addition, the reactive oxygen species (ROS) have been shown to be involved in apoptosis of luteal cells and addition of $\text{PGF}_{2\alpha}$ induced apoptosis by increasing the production of ROS [Sakka E et al, 1997]. In luteal cells, NO mediated $\text{PGF}_{2\alpha}$ -induced apoptosis by inducing the expression of Fas, caspase-3, and DNA ladder formation, which suggested that NO might play role in structural luteolysis [Korzekwa AJ et al, 2006].

Chapter II: Statement of problem

The regression of CL is initiated by $\text{PGF}_{2\alpha}$ in most species studied, including cattle [McCracken JA et al, 1970; Niswender GD et al, 2000]. Because of this property, $\text{PGF}_{2\alpha}$ has been routinely used for synchronization of estrous. However, the limitation of this approach is that the sensitivity of CL to $\text{PGF}_{2\alpha}$ - induced luteolysis varies depending on the developmental stage of CL. Specifically in cattle, the early developmental stages (on or before day-5) of CL is insensitive to the luteolytic actions of $\text{PGF}_{2\alpha}$ and the aging CL has increased sensitivity to $\text{PGF}_{2\alpha}$ compared to the early CL [Choudhary E et al, 2005; Copelin JP et al, 1988; Inskip EK, 1973; Sayre BL et al, 2000; Watts TL and Fuquay JW, 1985]. Therefore it is clear that: 1) $\text{PGF}_{2\alpha}$ is the initiator of luteolysis in the cattle, and 2) the sensitivity of CL is increased with developmental aging. However, the cellular mechanisms responsible for this developmental differences to $\text{PGF}_{2\alpha}$ is not clearly understood.

Several ideas have been proposed to be part of mechanisms responsible for developmental sensitiveness of CL as follows. 1) Alterations in the luteal expression of components associated with $\text{PGF}_{2\alpha}$ metabolism. For instance, resistance of early ovine CL has been attributed to increased expression of the $\text{PGF}_{2\alpha}$ catabolizing enzyme, hydroxyprostaglandin dehydrogenase 15-(NAD) (*HPGD*) [Silva PJ et al, 2000]. The inability of $\text{PGF}_{2\alpha}$ to induce the expression of prostaglandin-endoperoxide synthase 2 (*PTGS2*) and subsequent intraluteal $\text{PGF}_{2\alpha}$ synthesis in the early bovine CL was implicated in luteolytic insensitivity to $\text{PGF}_{2\alpha}$ [Tsai SJ and Wiltbank MC, 1998]. 2) Differences in signal transduction due to differential expression of genes associated with $\text{PGF}_{2\alpha}$ receptor. For example, increased expression of protein kinase C inhibitor-1 (PKCI-1) [now known as histidine triad nucleotide binding protein, (HINT1)] and kinase C inhibitor protein-1 (KCIP-1) [now known as tyrosine 3monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)] were reported to be involved in insensitivity of the early ovine CL [Juengel JL et al, 1998]. Greater expression of PRKCE in D-10 bovine CL has been proposed to participate in acquisition of luteolytic sensitivity to $\text{PGF}_{2\alpha}$ [Sen A et al, 2005]. 3) Alterations in the expression of locally produced hormones/factors that mediate the anitsteroidogenic actions of $\text{PGF}_{2\alpha}$. Inability of $\text{PGF}_{2\alpha}$ to induce eNOS in the early

bovine CL and subsequent absence of acute increase in luteal blood flow has been proposed be mechanism of early luteal resistance [Shirasuna K et al, 2008]. $\text{PGF}_{2\alpha}$ caused an acute increase in luteal blood flow during the initial stages of luteolysis and this event has been proposed to be due to ability of $\text{PGF}_{2\alpha}$ to induce vasodilator eNOS. In addition, low expression of endothelin converting enzyme-1 (ECE-1) and EDN1 peptide in early CL and inability of $\text{PGF}_{2\alpha}$ to induce EDN1 in early CL has been proposed to be one of the mechanisms responsible for refractoriness of early CL [Levy N et al, 2001; Levy N et al, 2000]. 4) The ability of the early CL to preferentially produce greater amount luteo-protective PGE2. The expression of PGES was greater in the early CL and decreased in regressing CL [Arosh JA et al, 2004]. In addition, the ability of PGES to convert PGH2 to PGE2 was 150-fold greater than conversion of PGH2 to $\text{PGF}_{2\alpha}$ by PGFS, there by producing greater amounts of PGE2 [Madore E et al, 2003]. PGE2 has been shown to be luteo-protective by promoting progesterone synthesis. In addition, $\text{PGF}_{2\alpha}$ has stimulated its own synthesis in CL via $\text{PRKC}/\text{Ca}^{2+}$ pathways by inducing the expression of COX-2 [Tsai SJ and Wiltbank MC, 1997]. Accordingly, the inability of $\text{PGF}_{2\alpha}$ to induce COX-2 in th early CL had been implicated in the mechanism of luteolytic sensitivity [Tsai SJ and Wiltbank MC, 1998].

Sen et al [Sen A et al, 2004] analyzed the expression pattern of array of PKC specific isozymes at two developmental (D-4 and -10) stages of bovine CL. The expression of PRKCE was greater in D-10 CL compared to D-4 CL. This observation led these authors to propose that the differential expression of PRKCE as a function of development could play a role in the observed transitional resistance/susceptibility to $\text{PGF}_{2\alpha}$ -induced luteal regression. In addition, specific PRKCE inhibitors abrogated the increase in $[\text{Ca}^{2+}]_i$ stimulated by $\text{PGF}_{2\alpha}$, [Sen A et al, 2005]. Moreover, the magnitude of intracellular Ca^{2+} signal stimulated by $\text{PGF}_{2\alpha}$ is greater in D-10 CL compared to early CL [Choudhary E et al, 2005; Sen A et al, 2005]. Therefore the ability of $\text{PGF}_{2\alpha}$ to stimulate greater magnitudes of $[\text{Ca}^{2+}]_i$ coincides with an increased expression of PRKCE. Therefore it was proposed that regulation of $[\text{Ca}^{2+}]_i$ might be a cellular mechanism through which PRKCE could mediate the inhibitory actions of $\text{PGF}_{2\alpha}$ on progesterone synthesis.

Further, effect of intracellular Ca^{2+} on steroidogenesis appears to be biphasic. In addition to inhibitory actions of high cytoplasmic Ca^{2+} on progesterone synthesis, the stimulatory effect of

Ca^{2+} on LH-stimulated progesterone has been reported. For example, absence of Ca^{2+} in culture media reduced the ability of LH/hCG to stimulate progesterone synthesis by 50% [Manna PR et al, 1999; Sullivan MH and Cooke BA, 1986]. In addition, Ca^{2+} ionophore A23187 stimulated hCG-stimulated progesterone synthesis and STAR expression in an additive manner [Manna PR et al, 1999], which indicated that effect of Ca^{2+} is not related to LH binding, but rather to cytoplasmic events. The stimulatory effect of Angiotensin II (Ang II) on adrenal steroidogenesis was associated with PLC activity and subsequent generation of IP_3 and mobilization of intracellular Ca^{2+} [Barrett PQ et al, 1989]. The stimulatory effect of Ca^{2+} on LH-stimulated progesterone is implied in the observations that a luteotrophic hormone increased IP_3 , and $[\text{Ca}^{2+}]_i$ in bovine luteal cells and porcine granulosa cells [Alila HW et al, 1990; Davis JS et al, 1987a; Flores JA et al, 1998]. Furthermore, $\text{PGF}_{2\alpha}$ stimulated LH-stimulated progesterone synthesis in the early bovine CL [Choudhary E et al, 2005; Levy N et al, 2001]. Therefore, the magnitude of Ca^{2+} signal stimulated by an agonist through its receptor might determine if the cellular response is luteolytic or luteotrophic at given developmental stage. The important implications of this interpretation is that by simply raising $[\text{Ca}^{2+}]_i$ to the appropriate threshold, one might be able to reduce progesterone regardless of the luteal developmental stage.

More importantly, lack of functional $\text{PGF}_{2\alpha}$ receptors does not seem to explain the mechanism of luteal insensitivity, because early CL (by Day-2) already express $\text{PGF}_{2\alpha}$ receptors with equal affinity and concentrations as observed in mature CL [Wiltbank MC et al, 1995]. Supporting this observation, $\text{PGF}_{2\alpha}$ can elicit distinct physiological responses in the early corpora lutea [Choudhary E et al, 2005; Levy N et al, 2000]. Therefore, the general idea upon which our current hypotheses were formulated was that the differences in components of signal transduction associated with luteal FP receptors at different developmental stages might be, at least in part, responsible for observed differences elicited by $\text{PGF}_{2\alpha}$. Furthermore, looking at the differential expression of single gene at a time might not be effective in unraveling the differences in complex signal transduction pathway associated with $\text{PGF}_{2\alpha}$ receptor. Therefore, studying global changes in the luteal transcriptome during its transition from early to mature stage might identify several genes that might play a role in developmental sensitiveness.

The hypotheses have been formulated based on the above discussed observations as follows:

Hypothesis 1a: PRKCE-mediates the anti-steroidogenic actions of $\text{PGF}_{2\alpha}$.

This hypothesis was tested using siRNA strategy to down-regulate the expression of PRKCE in steroidogenic cells. The expectancy was that PRKCE-specific siRNA should downregulate significant amount of mRNA and protein of PRKCE. This should allow us to test the role of PRKCE in mediating the antisteroidogenic actions of $\text{PGF}_{2\alpha}$ on LH-stimulated progesterone synthesis. Under low cellular concentrations of PRKCE, we predicted that the ability of $\text{PGF}_{2\alpha}$ to inhibit LH-stimulated progesterone synthesis might be compromised.

Hypothesis 1b: PRKCE was necessary for the expression of key genes of prostaglandin synthesis/metabolism that would favor $\text{PGF}_{2\alpha}$ synthesis; whereas in PRKCE down-regulated cells, the expression of key genes of prostaglandin synthesis/metabolism would be such that synthesis of PGE2 would be favored. This hypothesis was tested by determining changes in the expressions of key prostaglandin metabolic genes in PRKCE-down-regulated cells by real-time PCR.

Hypothesis 2: The rise in $[\text{Ca}^{2+}]_i$ is the cellular mechanism through which $\text{PGF}_{2\alpha}$ inhibits luteal progesterone. We reason that if a pharmacological treatment (calcium ionophore, A23187) is used to increase $[\text{Ca}^{2+}]_i$, this should inhibit LH-stimulated progesterone synthesis with equal effectiveness, regardless of the developmental stage (D-4 or -10) of CL. In addition, if the $\text{PGF}_{2\alpha}$ -stimulated increase in $[\text{Ca}^{2+}]_i$ is prevented with an intracellular Ca^{2+} chelator (BAPTA-AM), then $\text{PGF}_{2\alpha}$ will not be able to inhibit progesterone secretion.

Hypothesis 3a: The differential gene expression during the developmental transition of corpora lutea from D-4 to D-10 might include genes encoding components of signal transduction pathways that might change the nature of the elicited response, or the luteal sensitivity to luteolytic actions of $\text{PGF}_{2\alpha}$. This hypothesis was tested by utilizing an array that contained 8,329 unique bovine genes that was developed by Center for Animal Functional Genomics (CAFG) at Michigan State University. The expected result was that at least a portion of genes on the array might be differentially expressed in D-10 CL that could potentially explain the differential sensitivity of the bovine CL to $\text{PGF}_{2\alpha}$.

Hypothesis 3b: Some of the selected genes that are differentially expressed during transition from D-4 to D-10 developmental stage might be responsive to *in vivo* $\text{PGF}_{2\alpha}$ treatment on the D-4 or -10 of the estrous cycle. We selected some differentially expressed genes based on the literature that indicated these genes might participate in $\text{PGF}_{2\alpha}$ signaling. This hypothesis was tested utilizing real-time PCR using gene specific primers. The expected result was that the expression of some of the selected genes might be altered (increased/decreased) by exogenous $\text{PGF}_{2\alpha}$ that might potentially explain their role in acquisition of luteolytic sensitivity to $\text{PGF}_{2\alpha}$.

Hypothesis 3c: CAMKK2 mediates the actions of increasing $[\text{Ca}^{2+}]_i$ stimulated by $\text{PGF}_{2\alpha}$ on inhibiting progesterone. Observations from our microarray results have indicated that the expression of CAMMK2 was more than two fold greater in D-10 CL compared to D-4 CL. Furthermore, our preliminary studies have indicated that exogenous $\text{PGF}_{2\alpha}$ increased the expression of CAMKK2. This increase in CAMKK2 occurred at a luteal developmental stage that is sensitive to $\text{PGF}_{2\alpha}$. More importantly, its increased expression coincides with the luteal developmental stage at which $\text{PGF}_{2\alpha}$ has ability to elicit a greater rise in $[\text{Ca}^{2+}]_i$ [Choudhary E et al, 2005]. In many biological system, the actions of raising $[\text{Ca}^{2+}]_i$ are mediated via calmodulin-dependent protein kinases. Therefore we chose to study the role of CAMKK2 during the antisteroidogenic actions of $\text{PGF}_{2\alpha}$. Our prediction was that if the activity of CAMMK2 is inhibited, then the ability of $\text{PGF}_{2\alpha}$ to inhibit the progesterone synthesis is compromised under *in vitro* conditions. This hypothesis was tested using commercially available STO-609, which has been shown to be CAMMK2 specific inhibitor [Tokumitsu H et al, 2002].

Chapter III: PKC epsilon and an increase in intracellular calcium concentration are necessary for PGF₂alpha to inhibit LH-stimulated progesterone secretion in cultured bovine steroidogenic luteal cells

Introduction

The corpus luteum (CL) is a transient endocrine gland whose primary secretory product is progesterone (P4). The life span of the CL and consequently the amount of P4 it secretes is regulated according to reproductive physiological status. Substances reducing P4 secretion and shortening the luteal life span are said to be luteolytic [McCracken JA et al, 1999; Niswender GD and Nett TM, 1994].

In most species, including human beings, PGF_{2α} is recognized as an important if not the main luteolytic factor [Arosh JA et al, 2004; Auletta FJ and Flint AP, 1988; Guthrie HD et al, 1978; Olofsson J et al, 1992; Pate JL, 1988; Patwardhan VV and Lanthier A, 1980; Rexroad CE, Jr. and Guthrie HD, 1979]. During the ovarian cycle, the transition from early to mid-luteal phase is associated with changes in resistance/ susceptibility to the luteolysin PGF_{2α}; in cows, the CL is resistant to exogenous PGF_{2α} prior to day 5 of the estrous cycle [Choudhary E et al, 2005; Copelin JP et al, 1988; Inskeep EK, 1973; Sayre BL et al, 2000; Silva PJ et al, 2000; Silvia WJ and Niswender GD, 1984; Tsai SJ and Wiltbank MC, 1997; Wiltbank MC et al, 1995]. The cellular basis controlling luteal function during these physiological transitions, although studied intensely, is incompletely understood.

In steroidogenic cells of the ruminant CL, PGF_{2α} activates its plasma membrane G-protein-coupled receptor, which in turn activates the membrane-bound phosphoinositide specific phospholipase C (PLC), yielding inositol 1,4,5 trisphosphate (IP3) and diacylglycerol [Davis JS et al, 1988]. Indeed, in bovine luteal cells, PGF_{2α} stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis and mobilized intracellular Ca²⁺ [Davis JS et al, 1987b]. Accordingly, calcium and PRKC have been shown to be the intracellular mediators of PGF_{2α} actions in luteal cells [Wiltbank MC et al, 1991]. The regulatory effects of intracellular calcium concentration ([Ca²⁺]_i) on progesterone might be biphasic as there is also evidence for a

calcium requirement to support P4 synthesis by bovine luteal cells and LH, a luteotrophic hormone, increases IP3, and $[Ca^{2+}]_i$ in bovine luteal cells and in porcine granulosa cells [Alila HW et al, 1990; Davis JS et al, 1987a; Flores JA et al, 1998]. Therefore, there might exist thresholds of $[Ca^{2+}]_i$ that support or inhibit P4 synthesis.

Choudhary et al, [Choudhary E et al, 2005] tested the ability of increasing concentrations of $PGF_{2\alpha}$ to increase the $[Ca^{2+}]_i$ in large (LLC) and small (SLC) bovine luteal cells as function of development. Day-10 steroidogenic cells were more responsive to $PGF_{2\alpha}$ than Day-4 cell. Response amplitudes and number of responding cells were significantly affected by agonist concentration, luteal development and cell type. Response amplitudes were greater in LLC than in SLC; responses of maximal amplitude were elicited with lower agonist concentrations from Day-10 than from Day -4 cells. Furthermore, on Day-10, as concentrations of $PGF_{2\alpha}$ increased, larger percentages of SLC responded. Based on those results Choudhary et al proposed that the lower efficacy of $PGF_{2\alpha}$ in the early CL was likely related to signal transduction differences associated with the $PGF_{2\alpha}$ receptor at those two developmental stages [Choudhary E et al, 2005].

The array of PKC isozymes expressed in whole bovine CL includes α , βI , βII , ϵ and μ [Davis JS et al, 1996; Orwig KE et al, 1994; Sen A et al, 2004; Sen A et al, 2005]; and it has been demonstrated that the amount of $PKC\epsilon$ (PRKCE) expressed in the Day10 CL is greater than in the Day-4 CL [Sen A et al, 2004]. The latter observation led Sen et al, to propose that differential expression of PRKCE as a function of development could play a role in the observed transitional resistance/susceptibility to $PGF_{2\alpha}$ -induced luteal regression [Sen A et al, 2004; Sen A et al, 2005]. Sen et al, had further hypothesized that regulation of $[Ca^{2+}]_i$ was a cellular mechanism through which PRKCE could mediate actions of $PGF_{2\alpha}$ on P4 secretion [Sen A et al, 2005]. Additionally, there is evidence indicating that when bovine follicular theca cells are isolated and their luteinization is induced under in vitro tissue culture conditions, they express $PKC\delta$ [Budnik LT and Mukhopadhyay AK, 2002]. As $PKC\delta$ has been reported to play an important role in other species such as in rabbits and rodents [Maizels ET et al, 1996; Peters CA et al, 2000], this PKC isozyme might also be important for the physiology of the bovine ovary.

Endothelial cells of the bovine CL do not express PRKCE, although they do express the other PRKC isozymes described in the bovine CL [Sen A et al, 2006]. Data obtained with Western blot and immunohistological assays indicated that steroidogenic cells are the main source of PRKCE in the bovine CL [Sen A et al, 2006]. Therefore, in experiment 1, in order to assess the potential physiological role of PRKCE, we have used a siRNA strategy to down-regulate the expression of this PKC isozyme in luteal steroidogenic cells. In experiment 2, we used the PRKCE down-regulated cells to test two hypotheses. Our first working hypothesis was that PRKCE expression was necessary for $\text{PGF}_{2\alpha}$ to inhibit LH-stimulated P4 secretion in vitro. The second working hypothesis was that PRKCE was necessary for the expression of key genes of prostaglandin synthesis/metabolism that would favor $\text{PGF}_{2\alpha}$ synthesis; whereas in PRKCE down regulated cells, the expression of key genes of prostaglandin synthesis/metabolism would be such that synthesis of PGE2 would be favored. Finally, in experiment 3, we tested the hypothesis that $[\text{Ca}^{2+}]_i$ is the cellular mechanism through which $\text{PGF}_{2\alpha}$ inhibits luteal progesterone. We reasoned that if a pharmacological treatment is used to increase $[\text{Ca}^{2+}]_i$, this should inhibit luteal progesterone secretion with equally effectiveness, regardless of the developmental stage of the CL. Therefore, we used a pharmacological agent to increase $[\text{Ca}^{2+}]_i$ and examine its effects on LH-induced P4 secretion in luteal cells collected from early (Day-4) and mid-cycle (Day-10) bovine CL. Furthermore, this hypothesis was also tested by using a pharmacological agent to buffer any increase in $[\text{Ca}^{2+}]_i$ and examine, under conditions of low $[\text{Ca}^{2+}]_i$, the anti-steroidogenic effect of $\text{PGF}_{2\alpha}$ on LH-induced P4 secretion in cultures of luteal cells collected from mid-cycle (Day-10) CL.

Methods

Tissue collection

Non-lactating beef (experiments 1 and 2) or dairy (experiment 3) cows were observed visually for estrus twice daily at approximately 12-h intervals for a minimum of 30 min per observation. The day when standing estrus was observed was designated as Day 0 [Casida LE, 1959]. For experiments 1 and 2, the CL from four beef cows on Day-6 of the estrous cycle were collected in ice-cold saline and transported to the laboratory for luteal cell dispersion as

described below. For experiment 3, 14 non-lactating dairy cows were synchronized with 25 mg PGF_{2α} analog (Lutalyse®; Pfizer Animal Health., New York, NY) and ovaries on Day-4 (n = 4) or CL on Day-10 (n = 10) were collected surgically as described below and transported to the laboratory in ice-cold saline for dissociation and luteal cell enrichment as described below. The surgical procedure was performed via supravaginal incision under epidural anesthesia. For the epidural anesthesia, 6–9 ml 2% lidocaine was administered for cows weighing 450–700 kg (Butler Company, Columbus, OH). After surgery, penicillin (300,000 units) was administered intramuscularly to protect against post-surgical infection. The CL or ovary was collected into ice-cold saline at pH 7.4 and transported to the laboratory within 15 to 30 min after collection. The Animal Care and Use Committee of West Virginia University approved all procedures for these experiments (ACUC protocol # 060401).

Luteal cell dispersion and purification

In the laboratory, the CL was dissected free of connective tissue, weighed, placed into cell dispersion medium (CDM, M-199 containing 0.1% BSA, 25 mM HEPES, 100 U/ml fungicide), and cut into small (about 1 mm³) fragments. The tissue fragments were processed for tissue dissociation as previously described [Choudhary E et al, 2005]. Luteal endothelial cells were separated by a procedure previously described [Choudhary E et al, 2005; Levy N et al, 2001; Mamluk R et al, 1998; Webb BL et al, 1997]. Briefly, magnetic tosylactivated beads (Dynal Biotech, Lake Success, NY) were used to separate endothelial cells and the non-adherent cells, steroidogenic enriched luteal cells) were collected. The cell population designated as steroidogenic cells represented a heterogeneous population of cells including fibroblasts, pericytes, lymphoid and possibly few endothelial cells not removed by the separation procedure. Cell viability and density were determined using Trypan Blue exclusion and a hemocytometer; luteal cell viability was usually greater than 96%.

Experiment 1. Validation of siRNA methodology for specifically downregulating PRKCE expression in enriched steroidogenic luteal cells.

Day-6 dissociated luteal steroidogenic cells were cultured overnight at a cell density of 1×10^6 cells/well in 35 mm 24 – well culture dishes (Corning Inc, Corning NY) containing 1 ml Medium 199 supplemented with 5% fetal calf serum (FCS, GIBCO) at 37°C (95% air, 5%

CO₂). The next day cells were transiently transfected with PRKCE-specific siRNA kit (Upstate Cell Signaling solutions, Lake Placid NY) using lipofectin 2000 kit (Invitrogen Life Technologies) following the procedure recommended by the manufacturer. After transfection for 4 hr, the cultures were provided with M199 supplemented with 10% FCS, and incubated for a total of 48, 72 or 96 hours. After each of these time points, the cells were collected by adding 2 ml M199 containing 0.25% trypsin (GIBCO) to cover the monolayer and leaving the culture dish for about 1 min at room temperature. The cells were aspirated and washed one time with M199 containing 5% FCS and once with M199 without FCS. Cells collected from duplicate wells were pooled and the efficiency of transfection at 48, 72 and 96 h was analyzed by RT-PCR and Western blot analysis. Control groups included cells cultured in presence of M199 alone, M199 and transfecting reagent, and cells treated with non-specific siRNA duplex (non-specific siRNA).

Experiment 2. Effects of down-regulating PRKCE expression by the siRNA protocol on: A) the ability of PGF_{2α} to inhibit the LH-stimulated P4 accumulation, and B) on the expression of key genes involved in prostaglandin synthesis and metabolism.

Hypothesis 1: PRKCE is necessary for PGF_{2α} to be able to inhibit P4 secretion. To examine the ability of PGF_{2α} (Cayman Chemical, Ann Arbor, MI) to inhibit LH-induced progesterone accumulation, the siRNA transfected and control cells (not treated with PRKCE siRNA) were treated, after 96 h, with 100 ng/ml of LH, 1000 ng/ml of PGF_{2α}, or a combination of LH and PGF_{2α} for 4 hrs. After this time, the cell free medium was collected from each treatment and frozen until determination of P4 by radioimmunoassay (RIA). The RIA used for measurements of P4 in the culture media has been described previously [Sheffel CE et al, 1982]. The standard curve for this RIA ranged from 10 pg/ml to 800 pg/ml, and the intra- and interassay coefficients of variation were 9.2% and 12.8%, respectively.

Hypothesis 2: PRKCE is necessary for the expression of key prostaglandin biosynthetic/metabolizing enzymes. For the real time quantitative determination of gene expression of key prostaglandin biosynthetic/metabolizing enzymes in PRKCE down-regulated and control (not downregulated) cells, RNA samples were obtained from the cells collected in the experiment described under *Hypothesis 1*. The genes examined were: aldoketoreductase 1B5

(AKR1B5), prostaglandin-15 dehydrogenase (PGDH), prostaglandin E synthase (PGES), 9-keto-prostaglandin reductase (9K-PGR), and cyclooxygenase-2 (COX-2). Enriched steroidogenic cells were treated with the PRKCE siRNA protocol and after 96 h of culture the cells were treated with LH (100 ng/ml), $\text{PGF}_{2\alpha}$ (1000 ng/ml), or a combination of LH and $\text{PGF}_{2\alpha}$ for 4 h. The cells were collected by a brief trypsin treatment and total RNA was isolated with Trizol reagent according to the manufacturer's instructions (GIBCO). Total RNA was quantified spectroscopically at 260 nm and integrity of the RNA was determined by 2% agarose gel electrophoresis. Specific primers were designed by using primer3 software. The primer sequences and their accession numbers are shown in table 1. The single-step RT-PCR was carried out and cDNA product for each gene was column purified. Ten-fold serial dilutions of cDNA for each of the genes were used as templates to generate standard curves. Total RNA samples were reverse transcribed and used as templates in an iQ5 cycler (Bio-Rad Laboratories, Hercules, CA). The 25 μl reaction mixture contained 12.5 μl SYBER green mix (BioRad Laboratories), 2 μl cDNA sample, 2.5 μl each sense and antisense primers (0.5 μmol) and 5.5 μl of RNase free H₂O. The standard curves of threshold cycle (ct value) versus log starting quantity for the genes of interest were obtained. The conditions used were as follows: inactivation of RT enzyme, 95°C/3 min; denaturation, 95°C/30 sec; annealing, 55°C/30 sec; and extension, 72°C/1 min with fluorescence acquisition. The melt-curves were generated from 55°C to 95°C with 0.5°C increments in temperature. The melt-curves were observed for presence of single amplification product. The slope and intercept values obtained from the standard curve were used to determine the starting quantity for each gene using linear regression equation and gene expression for the desired gene was normalized using β -actin as the reference gene.

Experiment 3. The working hypothesis was that a rise in $[\text{Ca}^{2+}]_i$ is the cellular mechanism through which $\text{PGF}_{2\alpha}$ inhibits luteal P4.

Effect of a pharmacological increase in $[\text{Ca}^{2+}]_i$ on the LH-stimulated P4 secretion in Day-4 and -10 luteal steroidogenic cells. We predicted that if $[\text{Ca}^{2+}]_i$ is increased by a pharmacological treatment, this increase in $[\text{Ca}^{2+}]_i$ should be equally effective in reducing the LH-stimulated P4 secretion regardless of the developmental stage of the CL. The enriched steroidogenic cells (1×10^5 cells/well) isolated from Day-10 and Day-4 CL of $\text{PGF}_{2\alpha}$ -

synchronized non-lactating dairy cows were cultured overnight in 15 mm 24 -well culture plates in medium M199 supplemented with 0.1% BSA and 0.5% FCS. The next morning, the cells were treated in duplicate wells for 24 hr with M199 (control), LH (100 ng/ml), PGF_{2α}, (1.0 μg/ml), and a combination of LH and PGF_{2α}. The ability of increasing concentrations of the calcium ionophore, A23187 (0.1, 1, 10, or 100 μmol, (Invitrogen Detection Technologies), to inhibit basal and LH -stimulated P4 synthesis/secretion was tested in duplicate wells. The medium for the control group contained 0.1% dimethylsulfoxide (DMSO, Pierce Rockport, IL), the solvent used for PGF_{2α} and A23187. The cell-free media were collected and frozen until later measurements of P4 by RIA. The concentrations of A23187 used were based on single-cell studies, in which a concentration of 1 μmol A23187 was usually effective in increasing [Ca²⁺]_i to values comparable to those seen when cells were stimulated with PGF_{2α} at a concentration of 1000 ng/ml. The concentration range used of the Ca²⁺ ionophore should assure a very good probability of eliciting a wide range in increases in [Ca²⁺]_i that would allow testing its effect on the LH-stimulated P4 synthesis/secretion in Day-4 and -10 steroidogenic cells.

If the PGF_{2α} -stimulated increase in [Ca²⁺]_i is prevented, PGF_{2α} will not be able to inhibit P4 secretion. This experiment examined the ability of PGF_{2α} to inhibit LH-stimulated P4 secretion in Day-10 luteal cells under conditions in which elevations in [Ca²⁺]_i were buffered. This was accomplished by testing the effect of 1,2-bis (2-aminophenoxy) ethane-N, N, N', N'-tetracetic acid tetrakis acetomethyl ester, Bapta-AM (Invitrogen Detection Technologies, Carlsbad, CA), an effective pharmacological agent known to buffer changes in [Ca²⁺]_i [Midzak AS et al, 2007; Nikonenko I et al, 2005]. The concentration range chosen, 0.1 to 1000 μmol, was based on preliminary single-cell studies indicating that at the concentration of 10 μmol, Bapta-AM effectively prevented the typical increase in [Ca²⁺]_i induced by PGF₂ in luteal steroidogenic cells. The enriched Day-10 steroidogenic cells (1 × 10⁵ cells/well) isolated as described above were cultured overnight in 15 mm 24 -well culture plates in medium M199 supplemented with 0.1% BSA and 0.5% FCS. The next morning, the cells were treated in duplicate wells for 24 hr with M199 (control), LH (100 ng/ml), PGF_{2α}, (1.0 μg/ml), and a combination of LH and PGF_{2α} with increasing concentration of Bapta-AM (0.1, 1, 10, 100 or 1000 μmol). The effect of each treatment on basal and LH -stimulated P4 synthesis/secretion was tested in duplicate wells. The medium for the control group contained 0.1% dimethylsulfoxide (DMSO, Pierce Rockport, IL), the solvent used for PGF_{2α} and Bapta. The cell-free media were collected and frozen until later

measurements of P4 by RIA.

Semi-quantitative RT-PCR

The time-course effectiveness of the siRNA treatment in down-regulating PRKCE mRNA expression was determined by a semi-quantitative RT-PCR procedure (RT-PCR, Qiagen, Valencia, CA) previously validated and described [Orwig KE et al, 1994]. In this RT-PCR assay, PRKCE expression was normalized to the expression of GAPDH as the reference gene. The sequence of the PRKCE and GAPDH primers were those previously published: [Choudhary E et al, 2005], sense 5'-AGCTTGAAGCCCACAGCCTG-3'; antisense 5'-CTTGTGGCCGTTGACCTGATG-3'; and (34), sense 5'TGTTCCAGTATGATTCCACCC-3'; antisense 5'- TGTTCCAGTATGATTCCACCC-3' respectively. The specificity for these primer sets have been documented [Choudhary E et al, 2005; Orwig KE et al, 1994][Choudhary E et al, 2005; Orwig KE et al, 1994], and confirmed here by using the nucleotide database of National Center for Biotechnology Information with BLAST. The RT-PCR assay conditions were as follows: 50°C for 30min for reverse transcription reaction, 95°C for 15min for inactivation of RT enzyme, and then for PCR cycles consisted of 95°C for 50seconds for denaturing, 58°C for 30seconds for annealing, 72°C for 1min for extension and a final extension of 5min at 72°C. The RT-PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and viewed using the Fluro-S MultiImager (Bio-Rad Laboratories). Data were collected using densitometric analysis of Quantity One quantification software package (Version 4, Bio-Rad Laboratories). The intensity of the signal corresponding to PKC isozyme was standardized by the corresponding intensity of GAPDH control in that sample.

Semi-quantitative Western blots

Proteins were isolated from cells of siRNA treated and control groups using previously described methodology [Orwig KE et al, 1994]. Details for the semi-quantitative Western blot protocol used here have been described elsewhere [Sen A et al, 2004]. Briefly, protein samples (10 µg/lane) were resolved on an 8% polyacrylamide gel. The resolved proteins were transferred to polyvinylidene fluoride membrane (Biotechnology Systems, Boston, MA). The membranes were treated for immunodetection of the proteins of interest. The following

primary antibodies were used: a mouse anti-actin monoclonal antibody ([used at a dilution of 1:3000 (v/v)] Chemichon International, Inc., Temecula, CA); PKC isozyme specific (α , β I, β II, ϵ ,) polyclonal antibodies and their antigenic peptides ([antibodies used at dilution 1:1000] Gibco, Grand Island, NY). The following horseradish peroxidase-conjugated secondary antibodies were used here: anti-rabbit (1:5000, v/v; Amersham Pharmacia Biotech, and anti-mouse (1:30,000 v/v; GIBCO). Densitometry of the bands of interest were performed using Quantity One quantitation software. The intensity of the signal corresponding to the protein of interest was standardized by the corresponding intensity of the actin control in that sample. This normalization of data allows an estimate, in a semi quantitative manner, the amount of protein in the samples of interest, as described earlier [Sen A et al, 2004].

Statistics

The statistical software program from Statistical Analysis System, JMP 3.0 was used for data analyses [Cary NC]. Data were expressed as means \pm SEM for all the experiments. One-way ANOVA was used to determine effects of different treatments. Tukey – Kramer HSD was used to compare the different treatments subgroups. A value of $P < 0.05$ was considered statistically significant.

Results

Experiment 1

Culturing steroidogenic cells collected from the Day-6 CL spontaneously induced the expression of PRKCE (data not shown). Expression of PRKCE was induced gradually by the tissue culture conditions, and as Day-6 luteal cells were cultured up to 6 days, PRKCE expression had been spontaneously increased to values comparable to those seen in Day-10 CL (data not shown). Fig. 1A shows a typical result of the time-course siRNA experiments performed. The summarized data shown in Fig. 1B indicate that there was a significant ($P < 0.05$) decrease in the amounts of mRNA encoding PRKCE after 72 h of transfection (0.36 ± 0.07) compared to the media treated control group (1.03 ± 0.05). Panels A and B in Fig. 1 show that this approach reduced PRKCE expression 65 and 75% (0.23 ± 0.04) by 72 and 96 hrs of treatment respectively. This reduction was specific because no similar changes were observed

in treatments receiving only experimental media (Media), receiving non-specific siRNA duplexes (Non-Sp siRNA), or receiving only transfection reagents (Transfection reagent, Fig. 1B).

The effectiveness of the siRNA transfection in reducing protein corresponding to PRKCE can be seen in the semiquantitative western blotting (Fig 2A). A visual reduction in protein was detected 72 h after transfection (Fig. 2A). However, the semi-quantitative analysis of the data indicated that a significant reduction ($P < 0.05$) in the amount of PRKCE protein had not occurred until 96 h after transfection. At this time, there was a 50% reduction in the siRNA -treated group (0.39 ± 0.02) compared to control group (0.82 ± 0.07 , Media, Fig. 2B). Figure 3 demonstrates the specificity of the siRNA transfection in down-regulating the PRKCE isozyme. This Western blot was carried out for other PKC isozymes, PKC α and PKC β II, and there was no reduction in the amounts of these isozyme proteins even at 96 h after transfection with PRKCE-specific siRNA; a time by which there was significant reduction in PRKCE (Fig. 2B).

Experiment 2

Hypothesis 1: Effect of PRKCE down-regulation on the ability of PGF $_{2\alpha}$ to decrease the LH-induced P4 accumulation. Enriched steroidogenic cells ($n = 4$) transfected with PRKCE siRNA were cultured for 96 h and treated with LH, PGF $_{2\alpha}$, and combination of LH and PGF $_{2\alpha}$ for 4 h. The control group included cells treated with the hormones described above, but expressing normal amount of PRKCE. PRKCE down-regulation did not induce a decrease in the amount of P4 accumulation in the LH-stimulated cells (158.4 ± 18.1) compared to the control (202.4 ± 11.4). As in previous experiments, the accumulation of P4 was significantly reduced ($P < 0.05$) by PGF $_{2\alpha}$ (42.9 ± 2.6) compared to LH -treated control group (202.4 ± 11.4). There was no difference in the amounts of accumulated P4 between PGF $_{2\alpha}$ treated PRKCE down-regulated cells (34.9 ± 8.1) and control group (42.9 ± 2.6). However, the ability of PGF $_{2\alpha}$ to decrease LH-stimulated P4 accumulation was significantly ($P < 0.05$) inhibited in the PKC ϵ down-regulated group, 124.4 ± 7.4 compared to control, 51.4 ± 4.1 (Fig. 4).

Hypothesis 2: Gene expression of key prostaglandin biosynthetic/metabolizing enzymes in PRKCE down-regulated cells. Real-time PCR analysis of total RNA for mRNA encoding Cox-

2, AKR1B5, PGES, PGDH and PGE (2) -9-ketoreductase indicated that there were no significant differences in the expression of any of these genes as a functions of PRKCE down-regulation, LH or $\text{PGF}_{2\alpha}$ treatment (Fig. 7).

Experiment 3

A rise in $[\text{Ca}^{2+}]_i$ is the cellular mechanism through which $\text{PGF}_{2\alpha}$ inhibits luteal P4. Effect of a pharmacological increase in $[\text{Ca}^{2+}]_i$ on the LH-stimulated P4 synthesis/secretion in Day-4 and -10 luteal steroidogenic cells. As reported in previous studies [Choudhary E et al, 2005], basal P4 accumulation in cells collected form Day -4 CL was significantly lower than in those collected from Day -10 (7.6 ± 2.2 and 29.2 ± 1.8 respectively, Fig. 5A and 5B). LH significantly increased ($P < 0.05$) the luteal progesterone accumulation in both Day- 4 (49.5 ± 16.3) and -10 cells (65.7 ± 3.7). This effect of LH was not inhibited by $\text{PGF}_{2\alpha}$ in Day- 4 cells (44.6 ± 17.5), whereas it was significantly inhibited in Day-10 cells (31 ± 1.9 , Fig. 5A and 5B). When used at $0.1 \mu\text{mol}$, A23187 did not reduce LH-stimulated P4 accumulation in Day -4 or -10 cells; but at higher concentration ($1.0 - 100 \mu\text{mol}$), it negated the stimulatory effect of LH on P4 ($P < 0.05$, Fig. 5A – B). Basal P4 accumulation in Day -4 and -10 cells was not affected by any concentrations of A23187 tested (Fig. 5A and 5B, only $100 \mu\text{mol}$ A23187 shown).

If the PGF_2 -stimulated increase in $[\text{Ca}]_i$ is prevented, $\text{PGF}_{2\alpha}$ will not be able to inhibit P4 secretion. LH significantly increased ($P < 0.05$) the luteal progesterone accumulation in Day-10 cells (64.6 ± 3 , Fig. 6). This effect of LH was completely inhibited by $\text{PGF}_{2\alpha}$ (21.1 ± 2.1 , Fig. 6). Importantly, basal P4 accumulation (Fig. 6) was not affected by the Bapta-AM treatment, not even the highest concentration used (20.9 ± 4.1). When Bapta-AM was used at $0.1, 1, 10$ and $100 \mu\text{mol}$ in combination with LH, the values on P4 accumulation became intermediate between those observe for basal and LH alone (Fig. 6); and the stimulatory effect of LH was completely eliminated by $1000 \mu\text{mol}$ Bapta (data not shown). Consequently, the effect of Bapta-AM on the anti-steroidogenic action of $\text{PGF}_{2\alpha}$ could only be tested up to $100 \mu\text{mol}$. The inhibitory effect of $\text{PGF}_{2\alpha}$ on LH-stimulated luteal P4 accumulation was not affected by Bapta when used at concentrations not exceeding $1 \mu\text{mol}$, as the values for P4 accumulation clearly were not different from those observed for basal values ($P < 0.05$, Fig. 6). However, at

10 and 100 μmol , Bapta-AM effectively reduced the ability of $\text{PGF}_{2\alpha}$ to inhibit the stimulatory effect of LH on P4 accumulation (Fig. 6).

Discussion

The roles of specific PKC isozymes in luteal physiology have received little attention to date. As discussed below, these studies were designed to test the effects of ablating PRKCE expression in order to examine its hypothesized function. Previous studies had indicated that a potential function for PRKCE might be to regulate quantitatively the intracellular calcium signal initiated by $\text{PGF}_{2\alpha}$ on one of its luteal targets, the steroidogenic cells. The present studies validate the effective and specific down-regulation of PRKCE by siRNA technology and provide strong evidence about the function of this PKC isozyme in luteal physiology. The data support the overall hypothesis that downregulating expression of PRKCE reduces the effectiveness of $\text{PGF}_{2\alpha}$ in reducing progesterone secretion. This observation extends the report that when PRKCE was inhibited with PRKCE-specific inhibitors, the $\text{PGF}_{2\alpha}$ – induced rise in $[\text{Ca}^{2+}]_i$ was decreased in LLC and SLC and that this in turn had consequences (at least in part) in the ability of $\text{PGF}_{2\alpha}$ to inhibit LH-stimulated P4 secretion at this developmental stage [Sen A et al, 2005]. As previously reported [Choudhary E et al, 2005], LH induced an increase in the amount of P4 secretion. Interestingly, in the group where PRKCE expression was down regulated, the inhibitory effect of $\text{PGF}_{2\alpha}$ on LH-stimulated P4 secretion was significantly mitigated (Fig. 3). This observation has an important physiological corollary: both $\text{PGF}_{2\alpha}$ -receptors and PRKCE are expressed in the same luteal cell type. Therefore, the isozyme PRKCE has an important compatible time (mid-luteal phase) and place (small and large luteal steroidogenic cells) of expression, for it to have a role in the luteal transition from resistance to sensitivity to luteolytic actions of $\text{PGF}_{2\alpha}$. Furthermore, if PRKCE expression is down - regulated (this study) or if its activation is inhibited [Sen A et al, 2005], the anti-steroidogenic effect of $\text{PGF}_{2\alpha}$ on LH-stimulated P4 secretion is impaired.

Experiment 2 also tested the hypothesis that down-regulating PRKCE could influence the expression of key PG metabolizing enzymes that, in turn, could influence the balance of PG production from luteo-protective or luteotrophic to luteolytic. The mechanism for luteal

resistance is not exactly known. However there is now evidence that regulation of key PG metabolizing enzymes observed during physiological states in which the life span of the CL is modified is likely to play an important role in this complex process [Asselin E and Fortier MA, 2000; Asselin E et al, 1997; Griffeth RJ et al, 2002; Hu YF et al, 1990; Patek CE and Watson J, 1976; Rexroad CE, Jr. and Guthrie HD, 1979; Shemesh M and Hansel W, 1975a; Silva PJ et al, 2000; Xiao CW et al, 1998]. The selection of the examined genes was based on the available evidence that, because of their key positions in the PG biosynthetic pathway, these genes have been shown to determine the accumulation of luteolytic or luteotrophic classes of PG [Asselin E and Fortier MA, 2000; Asselin E et al, 1997; Patek CE and Watson J, 1976; Shemesh M and Hansel W, 1975a; Xiao CW et al, 1998]. For example, we examined the effects of down-regulating PRKCE on the expression of PGE2 and F synthases because of their more direct effect on determining whether PGH2 is metabolized to PGE2 or PGF_{2α}. The results obtained were unexpected; the prediction was that because of low expression of PRKCE, exogenous PGF_{2α} would not be able to induce high increases in the cytosolic concentration of calcium, and consequently, the expression of PGE2 synthase/PGF_{2α} synthase ratio would favor PGE2 synthesis. The above conditions would favor luteal function. However, it is worth pointing out the importance of looking beyond steady states of mRNA encoding these enzymes; sometimes regulation may be at the level of protein or even enzyme activity and additional work is necessary before rejecting the tested hypothesis.

The developmental significance of a regulatory role played by cytosolic calcium concentrations in mediating the inhibitory actions of PGF_{2α} is documented by results obtained in experiment 3. As reported in previous studies [Choudhary E et al, 2005], PGF_{2α} reduced LH-stimulated P4 secretion in Day10 cells only. Basal P4 secretion was not affected by the PGF_{2α}-treatment at any of the two developmental stages tested. As the working hypothesis predicted, the pharmacological increase in [Ca²⁺]_i induced by A23187 effectively mimicked the inhibitory effect of PGF_{2α} in Day -10 steroidogenic cells. Furthermore, as predicted by the working hypothesis, the A23187 treatment also inhibited LH-stimulated P4 secretion in Day -4 steroidogenic cells. This inhibitory effect of A23187 is most likely due to its demonstrated effect in increasing the intracellular concentration of calcium ions [Sen A et al, 2005] in these cells and not due to other non-specific effects. This interpretation is also supported by the observation that treatment with A23187 had no negative effect on basal P4 secretion at any of

the two developmental stages tested.

Further support for the significance of a regulatory role played by the increase in $[Ca^{2+}]_i$ in mediating the inhibitory actions of $PGF_{2\alpha}$ is documented by results obtained in experiment 3 where the cytoplasmic calcium buffering capacity of the cells was increased by Bapta-AM. At lower concentrations (0.1 and 1.0 μmol), the calcium buffering capacity of Bapta-AM was, most likely, at values that still allowed a $PGF_{2\alpha}$ -stimulated increase in $[Ca^{2+}]_i$; which in turn, preserved the ability of $PGF_{2\alpha}$ to inhibit LH-stimulated P4 secretion (Fig. 6). However, as the calcium buffering capacity in the cytoplasm of the steroidogenic cells was increased by increasing the concentration of Bapta AM (10 and 100 μmol), the calcium signaling feature of activating the $PGF_{2\alpha}$ receptors was most likely eliminated or at least reduced, and consequently, the ability of $PGF_{2\alpha}$ to inhibit LH-stimulated P4 secretion was also significantly reduced (Fig. 6). Similar effects of Bapta-AM on basal and hormonal-stimulated steroidogenesis have been reported in MA-10 Leydig cells (34). Therefore, the results of experiment 3 stress the calcium requirement for $PGF_{2\alpha}$ to inhibit LH-stimulated P4 secretion in the midphase CL and support the reported observation that the lower efficacy of $PGF_{2\alpha}$ to inhibit P4 secretion in the early CL is related to the reduced ability of $PGF_{2\alpha}$ to increase the cytoplasmic concentration of calcium at this developmental stage [Choudhary E et al, 2005]. Taken together, the results obtained in the A23187 and Bapta-AM experiments, strongly support the proposed hypothesis that an attenuation of the luteolytic actions of $PGF_{2\alpha}$ is associated with a compromise in the ability of $PGF_{2\alpha}$ to induce a rise in $[Ca^{2+}]_i$ [Sen A et al, 2005]. Therefore these studies provide a strong linkage between the signal transduction utilized by the $PGF_{2\alpha}$ receptor at different developmental stages and quantitative aspects of the known intracellular mediator of $PGF_{2\alpha}$ actions in the CL, $[Ca^{2+}]_i$. In this regard, species differences do exist, as in rat luteal cells the antigonadotropic action of $PGF_{2\alpha}$ is not mediated by elevated cytosolic calcium levels [Pepperell JR et al, 1989]. It appears that the bovine CL therefore, has the following commonalities with human CL: 1) in both species, $PGF_{2\alpha}$ is luteolysin, 2) the luteolytic effect of $PGF_{2\alpha}$ appears only during mid- and late-luteal phase, and 3) in both, the humans and cows, changes in intracellular calcium appear to regulate luteal function ([Ottander U et al, 1999] and this study).

In summary, the evidence presented here strongly supports the idea that PRKCE, an isozyme highly expressed in steroidogenic luteal cells with acquired luteolytic response to

PGF_{2α}, has an important regulatory role in the ability of PGF_{2α} to inhibit LH-stimulated P4 secretion in vitro at this developmental stage. The data presented strongly support the hypothesis that luteal resistance to the luteolytic actions of PGF_{2α} is associated with a compromised ability of PGF_{2α} to induce a rise in [Ca²⁺]_i. If the PGF_{2α} receptor and its associated signal transduction is bypassed with a pharmacological agent to increase the [Ca²⁺]_i, the LH-stimulated P4 secretion in Day-4 steroidogenic cells is eliminated, an action that cannot be induced by PGF_{2α} at this developmental stage. Conversely, if the increase in [Ca²⁺]_i typically induced by PGF_{2α} on Day-10 steroidogenic luteal cells is buffered by a pharmacological agent, then the ability of PGF_{2α} to inhibit the LH-stimulated P4 secretion is abrogated.

Table 1. Primer sequence, accession number, of investigated genes

Gene	Forward primer	Reverse primer	Acc#
<i>β-Actin</i>	GACATCCGCAAGGACCTCTA	ACGGAGTACTTGCGCTCAG	BC102948
<i>PGDH</i>	GGAAAGCTGGACATCTTGGT	GCAAATTGCGTTCAGTCTCA	BC102458
<i>PGES</i>	GAACGACCCAGATGTGGAA	ATACGGCCCAGGAAGAAGAC	NM174443
<i>AKR1B5</i>	GACCTTGGGTACCGTCACAT	TCTTTCTCACTGGGAATCACG	S54973
<i>9K-PGR</i>	AAGAAATGCAGCCGTGAACT	GCTCCTTCTTCTGGGCTTTT	BC102943
<i>COX-2</i>	CATGATGTTCTTTGTTGGCATT	GCGAATTCCAACCTTTCCATC	AF031698

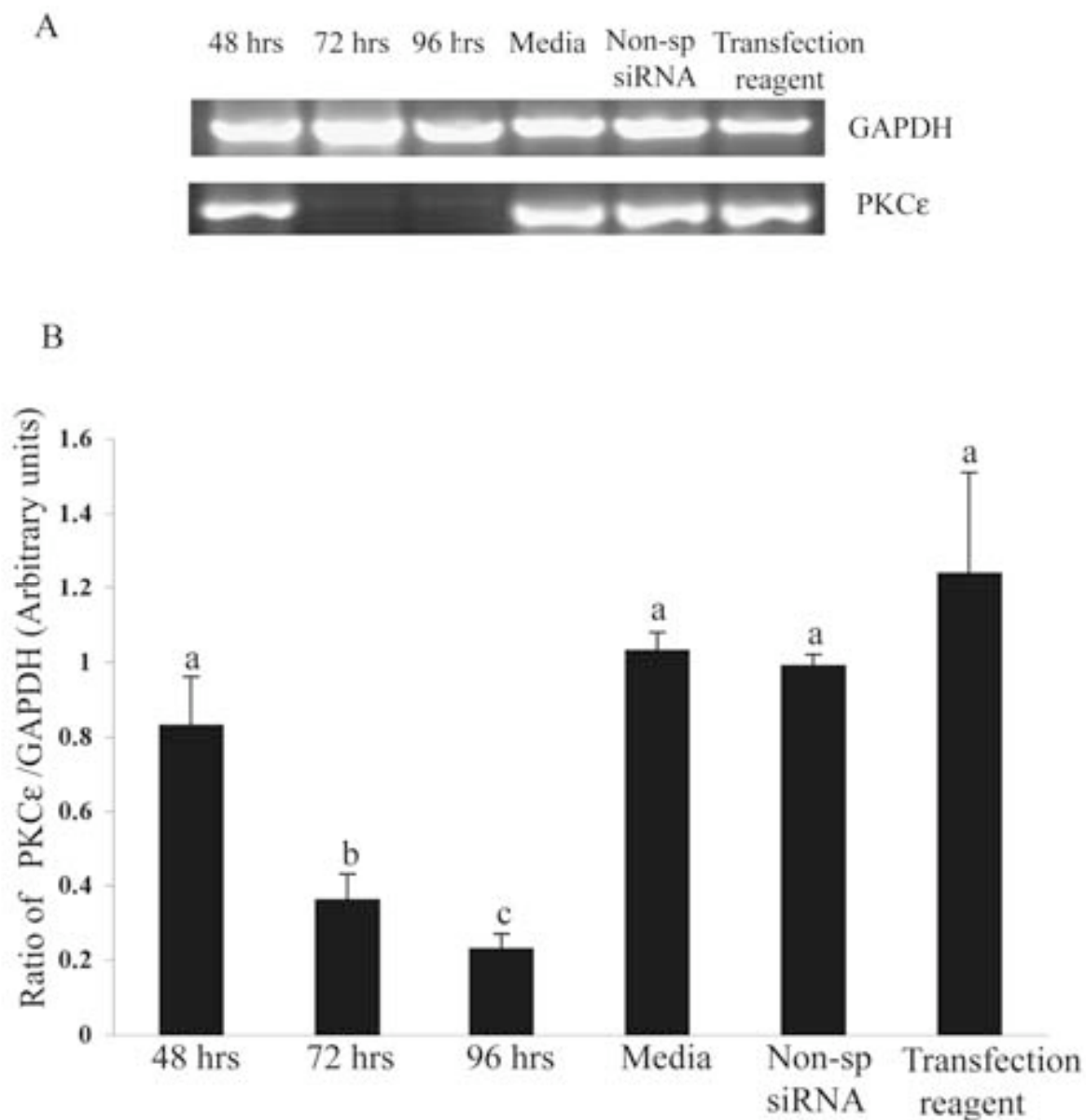


Figure 1. Time-course reduction in PKC ϵ (PRKCE) mRNA expression after transfection of luteal steroidogenic cells with PRKCE specific siRNA. (A) Representative RT-PCR products obtained from total RNA using the PRKCE and GAPDH primers. The amount of total RNA was adjusted to 200 ng per reaction and 40 cycles were used for PRKCE; while 28 cycles were used for GAPDH. The size of the amplified products for the GAPDH and PRKCE were 900 and 500 bp, respectively. PRKCE and GAPDH mRNA expression after 48, 72, and 96 h of transfection with PRKCE specific siRNA are shown. Lanes labeled media, non-specific (Non-sp) siRNA, and Transfection reagent represent respective treatments without PRKCE specific

siRNA treatment. GAPDH was used as the control gene to normalize the PRKCE mRNA expression. (B) Quantitative analysis of the RT-PCR products obtained in four ($n = 4$) replicates similar to those shown in panel A. Data are the mean \pm SEM of the densitometry measurements for PRKCE relative to GAPDH mRNA. Statistical comparisons were made between different treatments. Different letters above each SEM represent different values ($P < 0.05$).

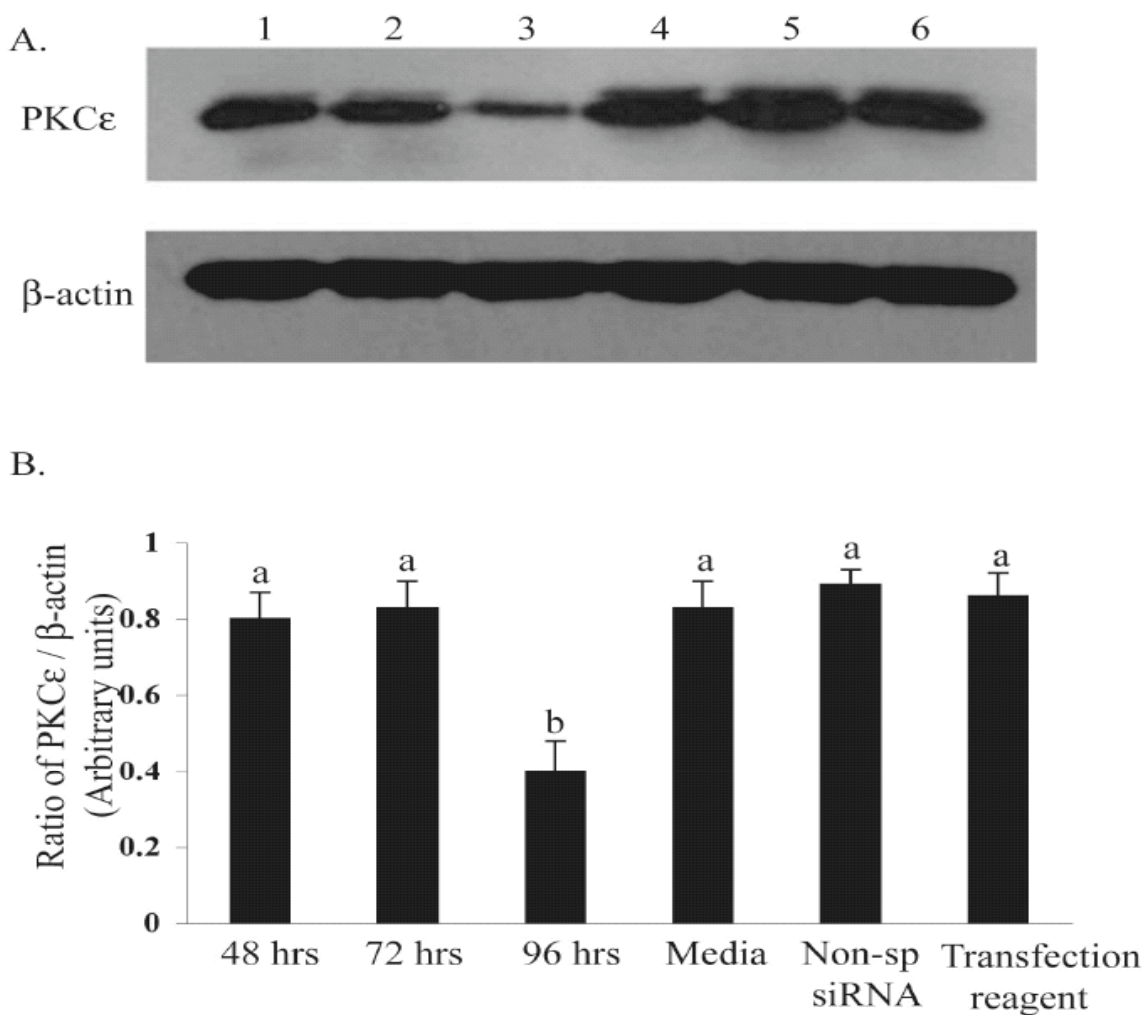


Figure 2. Reduction in PRKCE protein. (A) Representative Western blot showing the amount of PRKCE and actin expressed in protein samples prepared from luteal steroidogenic cells after 48, 72, and 96 h of transfection with PRKCE specific siRNA (lanes 1–3). Lanes labeled 4 – 6, contained protein samples from indicated control treatments (media, Non-sp siRNA, and transfection reagent, respectively). (B) Semi-quantitative analysis of the densitometry derived

from four experiments similar to the one shown in panel A. the y-axis shows the ratio of the optical density ratio of PKC ϵ to that of its corresponding β -actin. The data are shown as mean \pm SEM, and comparisons were made between different treatments. Values with different letters denote differences by one-way ANOVA followed by Tukey-Kramer honestly significant difference ($P < 0.05$).

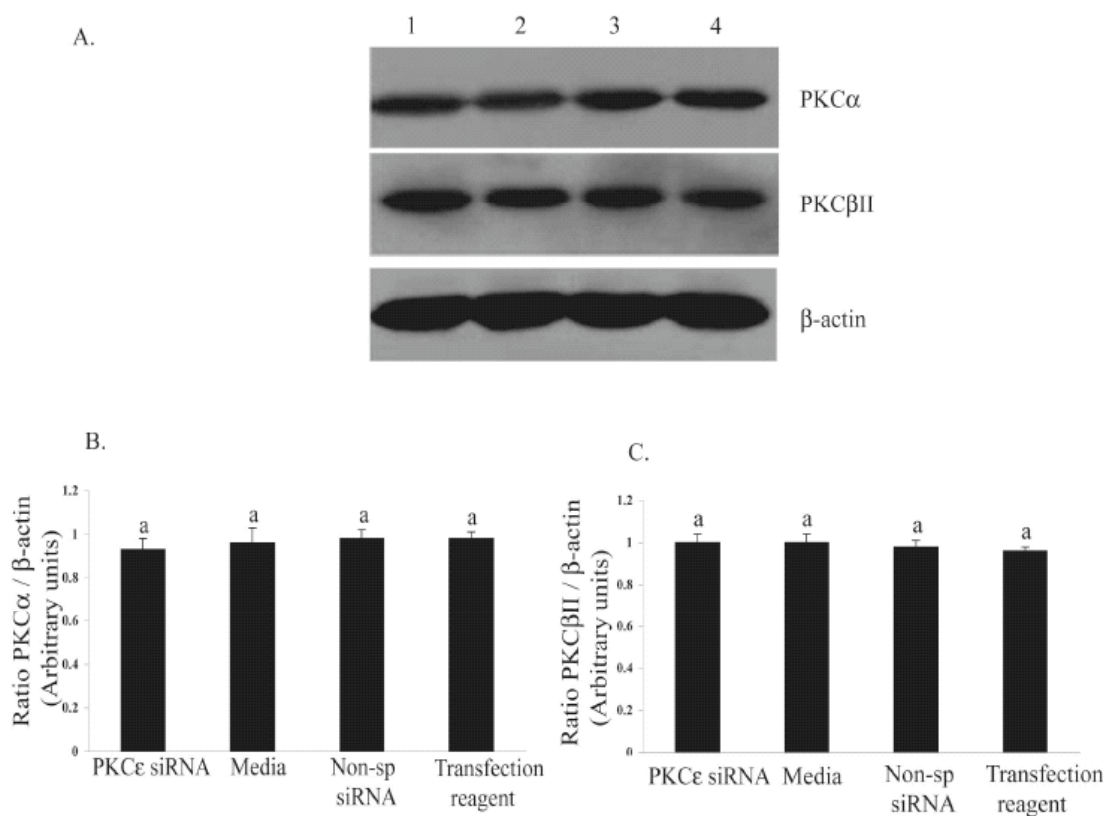


Figure 3. PKC α and PKC β II protein after 96 h transfection of luteal steroidogenic cells with PRKCE specific siRNA. (A) Representative Western blot showing the amount of PKC α , PKC β II KC ϵ and actin detected in protein samples prepared from luteal steroidogenic cells after 96 h of transfection with PRKCE specific siRNA (lane 1). Lanes labeled 2 – 4, contained protein samples from indicated control treatments (media, Non-sp siRNA, and transfection reagent, respectively). B and C) Semi-quantitative analysis of the densitometry derived from four experiments similar to the one shown in panel A for PKC α (B) and PKC β II (C). The y-axis shows the ratio of the optical density ratio of PKC isozyme to that of its corresponding β -actin. The data are shown as mean \pm SEM, and comparisons were made between different

treatments by one-way ANOVA followed by Tukey-Kramer honestly significant difference.

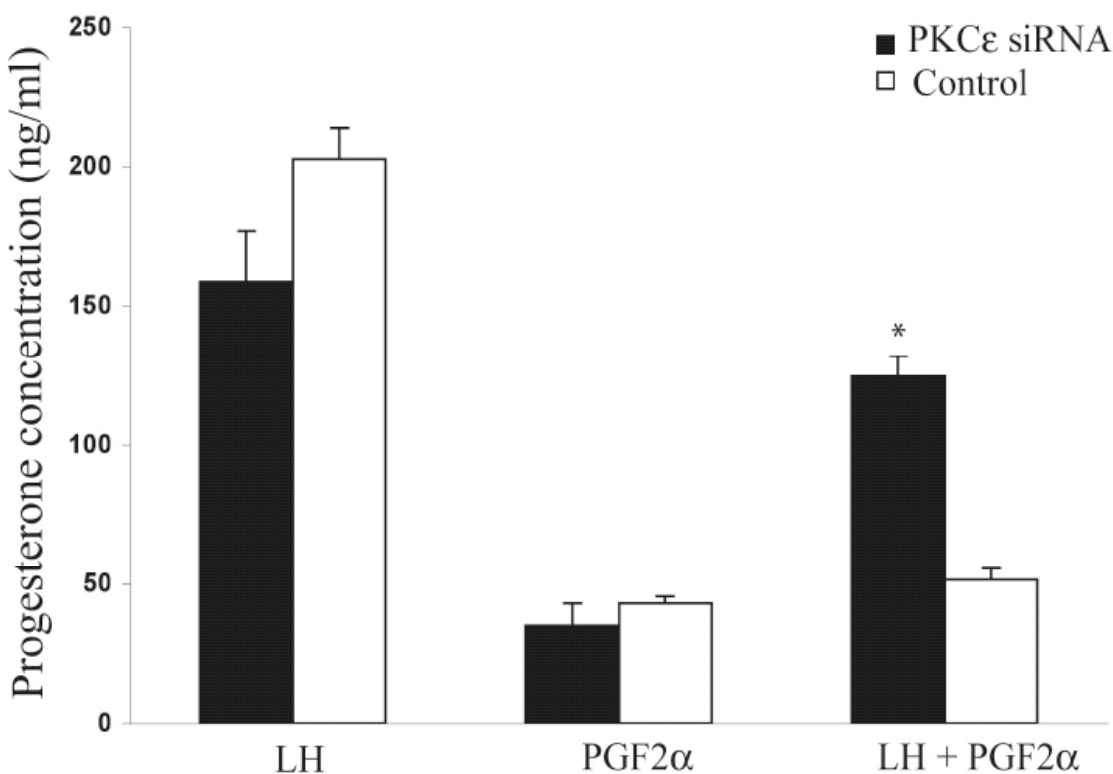


Figure 4. Effects of PRKCE down-regulation on the ability of PGF_{2α} to inhibit the LH-stimulated progesterone synthesis/secretion in cultures of steroidogenic luteal cells transfected for 96 h with PRKCE specific siRNA (filled bars) or with transfection reagents (control, open bars). Progesterone accumulation was determined in culture media after 4 h of incubation in the following treatments: LH (100 ng/ml), PGF_{2α} (1 μg/ml) and a combination of PGF_{2α} and LH. Data are presented as mean ± SEM of four individual replicates (n = 4 cows). For each treatment group, statistical comparisons were made between PRKCE down-regulated (PRKCE siRNA) and control (not PRKCE down-regulated); different letters above each SEM denote different values, P < 0.05.

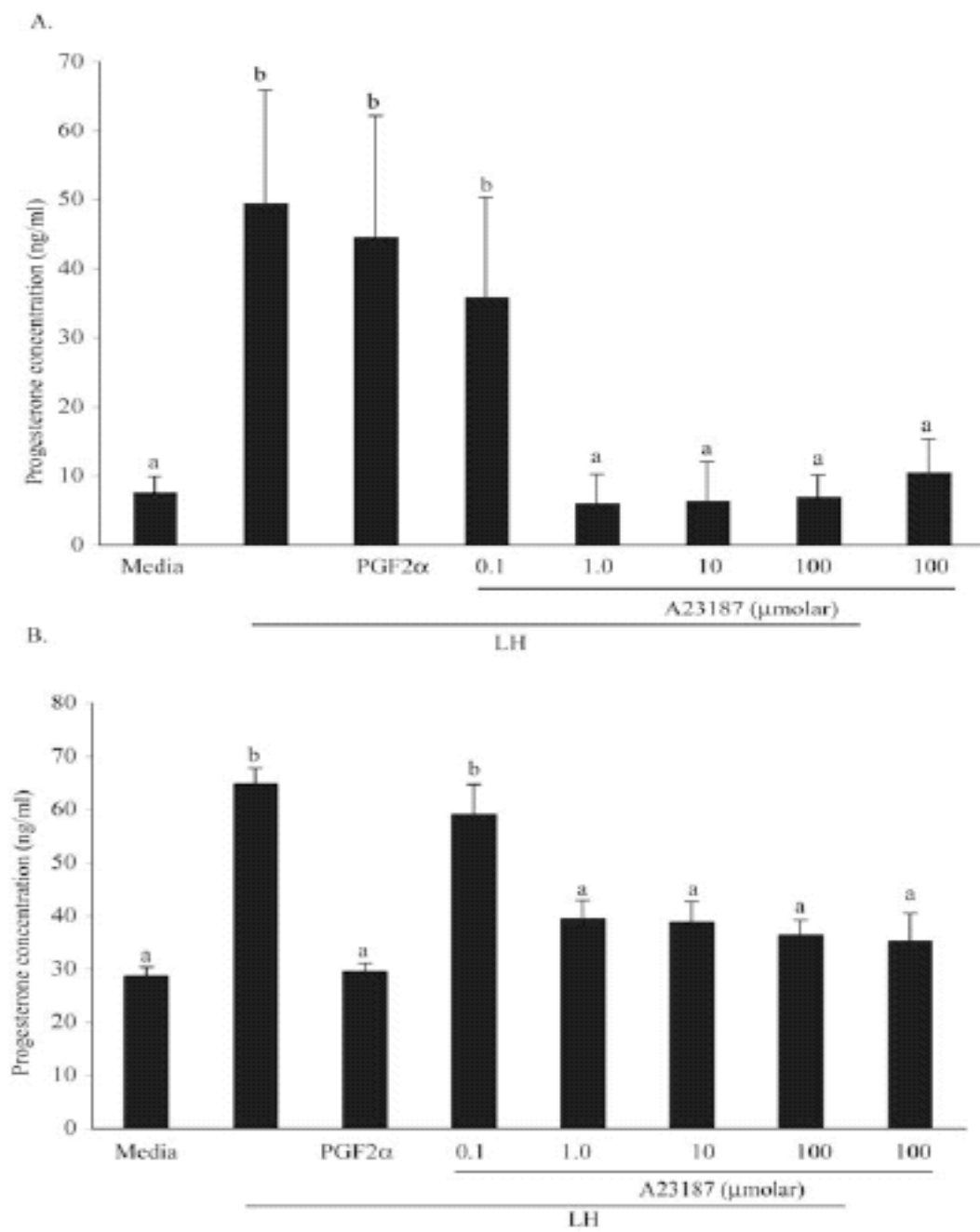


Figure 5. Effect of the Ca^{2+} ionophore, A23187, on basal and LH-stimulated progesterone synthesis/secretion (ng/ml) in cultured steroidogenic cells collected from Day 4 (panel A) and Day 10 (panel B) bovine CL. Progesterone accumulated in culture media was determined after 4 h of incubation in the following treatments: media alone (Media), LH (100 ng/ml), LH and $\text{PGF}_{2\alpha}$ (1000 ng/ml), or LH and A23187 (0.1, 1, 10, and 100 μmol). As explained in Materials and Methods, these treatments also contained 0.1% of the solvent used for $\text{PGF}_{2\alpha}$ and A23187, DMSO. Data are presented as the mean \pm SEM of four Day 4 and 10 Day 10 individual replicates ($n = 4$ and 10 cows respectively). Statistical comparisons were made across treatments, and means with different letters, differ within each panel ($P < 0.05$)

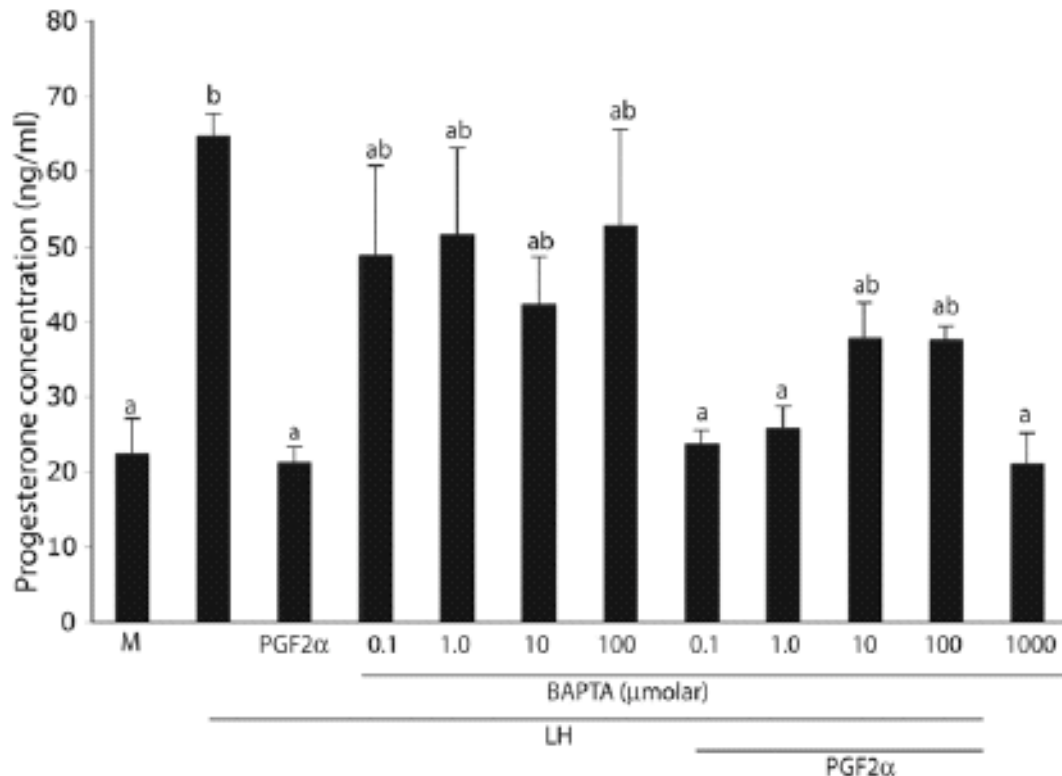


Figure 6. Effect of the cell-permeable calcium chelator, Bapta-AM, on basal and LH-stimulated progesterone synthesis/secretion (ng/ml) in cultured steroidogenic cells collected Day 10 bovine CL. Progesterone accumulated in culture media was determined after 4 h of incubation in the following treatments: media alone (Media), LH (100 ng/ml), LH and PGF_{2α} (1000 ng/ml), or LH and Bapta-AM (0.1, 1, 10, and 100 μmol). As explained in Materials and Methods, these treatments also contained 0.1% of the solvent used for PGF_{2α} and Bapta-AM, DMSO. Data are presented as the mean ± SEM of four Day 10 individual replicates (n = 4 CL obtained from 4 cows). Statistical comparisons were made across treatments, and means with different letters denote different values, P < 0.05.

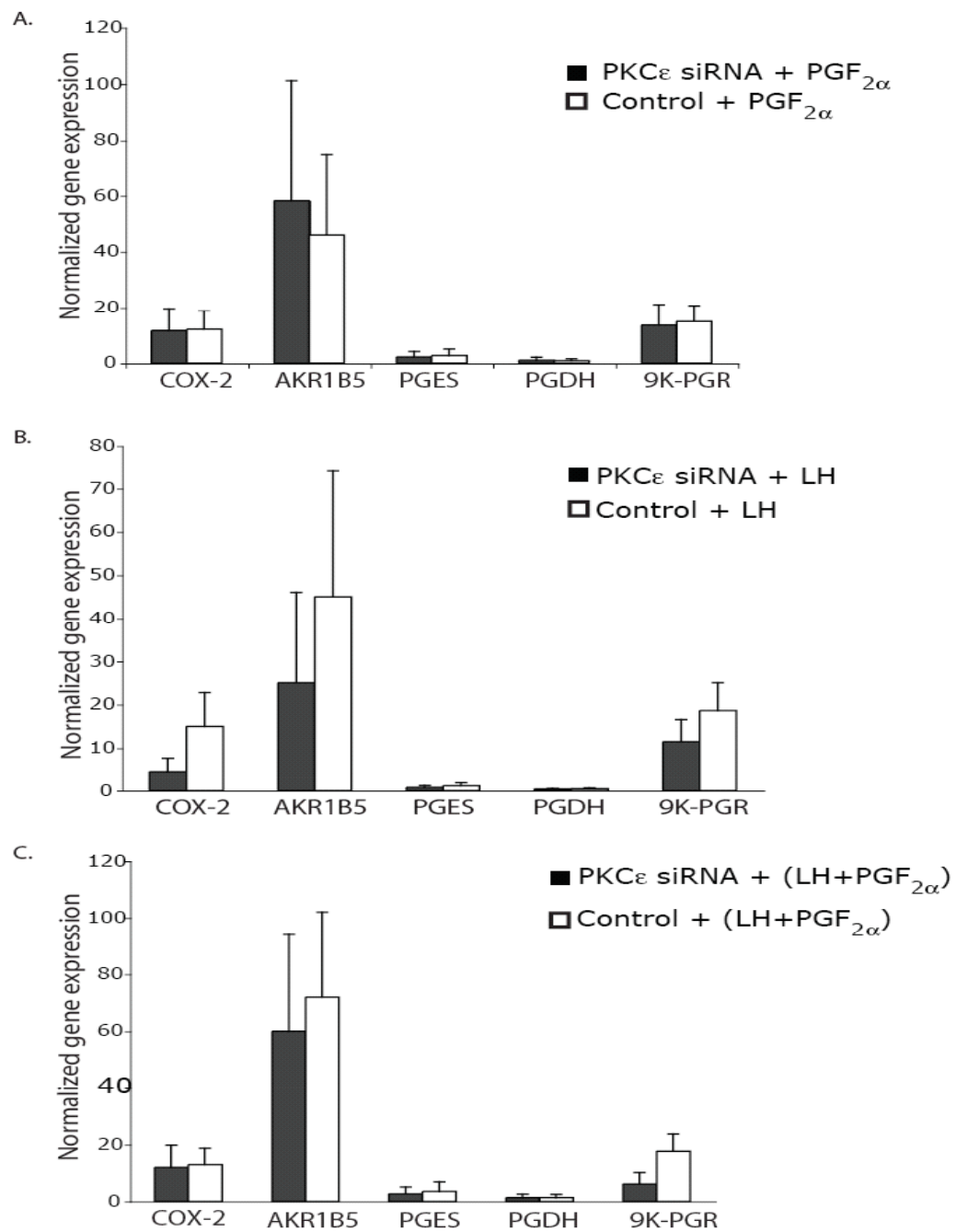


Figure 7. Real-time PCR analysis of the effect of down-regulation of PRKCE mRNA on the expression of PGF_{2α} metabolic genes. Comparisons of mRNA expression data were made between the control (without PRKCE down-regulation) and PRKCE down-regulated steroidogenic cells treated either with PGF_{2α} (**A**), LH (**B**), and combination of PGF_{2α} and LH (**C**). The genes analyzed were as follows: aldoketoreductase 1B5 (AKR1B5), prostaglandin-15 dehydrogenase (PGDH), prostaglandinE synthase (PGES), 9-keto-prostagalndin reductase (9K-PGR), and cyclooxygenase-2 (COX-2). The expression data were presented as mean ± SE. The comparison of means between treatments by t-test did not show significant differences with any of the genes analyzed.

Chapter IV: Differential Gene Expression in the Bovine Corpus Luteum During Transition from Early to Mid-Phase and Its Potential Role in Acquisition of Luteolytic Sensitivity to Prostaglandin F₂ Alpha

Introduction

Luteal regression is required for normal ovarian cyclic activity. Prostaglandin F_{2α} (PGF_{2α}) initiates luteal regression or luteolysis in cattle and most domestic species [McCracken JA et al, 1970; Niswender GD et al, 2000], and has been used for estrous synchronization in beef and dairy cattle [Inskeep EK, 1973; Lamb GC et al, 2006; Lauderdale JW et al, 1974; Silva E et al, 2007]. However, the bovine corpus luteum (CL) is resistant to luteolysis by exogenous PGF_{2α} prior to day 5 of the estrous cycle [Choudhary E et al, 2005; Copelin JP et al, 1988; Goravanahally MP et al, 2007; Inskeep EK, 1973; Sayre BL et al, 2000; Watts TL and Fuquay JW, 1985; Wiltbank MC et al, 1995]. The cellular basis controlling luteal function during this physiological transition, although studied intensely, is incompletely understood. As in many biological systems, cellular responsiveness to a given agonist can be altered during development. Lack of PGF_{2α} receptors does not seem to explain luteal insensitivity to PGF_{2α} as the developing CL already expresses high affinity PGF_{2α} (FP) receptors [Sakamoto K et al, 1994; Wiltbank MC et al, 1995], and PGF_{2α} can elicit distinct physiological responses in the early CL [Sayre BL et al, 2000; Sen A et al, 2005; Tsai SJ and Wiltbank MC, 1998]. Therefore the nature of the elicited response or the ineffectiveness of PGF_{2α} to induced luteolysis in developing CL might be due to differences in post-receptor signaling events. Several studies have indicated that the lower efficacy of PGF_{2α} in inducing regression of early CL might be related to differences in signal transduction due to differential expressions of genes associated with the FP receptor at those two developmental stages. For instance, increased expression of protein kinase C inhibitor-1 (now known as histidine triad nucleotide binding protein, (HINT1) and tyrosine 3monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) was reported to be involved in the insensitivity of the early ovine CL [Juengel JL et al, 1998]. Higher expression of PRKCE in D10 bovine CL has been reported to be involved in acquisition of sensitivity of the CL to PGF_{2α}-induced luteolysis [Sen A et al, 2005]. It has been suggested that the lack of luteolytic action by PGF_{2α} in the developing bovine CL might be due to alterations in components of the signal transduction associated with the receptor by locally

produced hormones. For instance, resistance of early ovine CL has been attributed to increased expression of the $\text{PGF}_{2\alpha}$ catabolizing enzyme, hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD) [Silva PJ et al, 2000]. The inability of $\text{PGF}_{2\alpha}$ to induce expression of prostaglandin-endoperoxide synthase 2 (PTGS2) and intraluteal $\text{PGF}_{2\alpha}$ synthesis in the early bovine CL was implicated in luteolytic insensitivity to $\text{PGF}_{2\alpha}$ [Tsai SJ and Wiltbank MC, 1998]. Therefore, the hypothesis tested was that differential gene expression during the developmental transition of corpora lutea from D-4 to D-10 might include genes encoding components of signal transduction pathways that might change the nature of the elicited response, or the luteal sensitivity to luteolytic actions of $\text{PGF}_{2\alpha}$. The two objectives of the present study were to 1) use a microarray-based approach to systematically analyze a large portion of the bovine CL transcriptome during the developmental transition from D-4 to -10, when the luteolytic capability to $\text{PGF}_{2\alpha}$ is acquired; and 2) determine the responsiveness of selected genes found to be differentially expressed during this transition to an exogenous *in vivo* treatment with $\text{PGF}_{2\alpha}$ on the D-4 or -10 of the estrous cycle.

Materials and Methods

Animal handling and surgical procedures

Non-lactating beef cows were observed visually for estrus twice daily at approximately 12-h intervals for a minimum of 30 min per observation. The day when standing estrus was observed was designated as Day 0. For experiment 1, ovaries on Day -4 (n = 3) or CL on Day -10 (n = 3) were collected surgically as described previously [Choudhary E et al, 2005] and transported to the laboratory in ice-cold saline for RNA isolation. Briefly, the surgical procedure was performed via supravaginal incision under epidural anesthesia (6-9 ml 2% lidocaine hydrochloride; Butler Company, Columbus, OH) administered for cows weighing 450 -700 kg. For experiment 2, beef cows on Day 4 or 9 of estrous cycle were treated with 25 mg of $\text{PGF}_{2\alpha}$ analog (Lutalyse; Pfizer Animal Health, New York, NY) and control groups received 5 ml of normal saline (n=3 per group per day). After 24h, Day -5 or -10 ovaries or, CL were collected as described above. For experiment 4, beef cows on days 10 (n=4) were utilized for CL collection. For experiment 5, corpora lutea from beef cows (n=4 per group) on days -4 and -10 were collected. The ovary or CL was collected into ice-cold saline (PBS) and

transported to the laboratory within 15 to 30 min after collection. Developing corpora lutea were removed from the ovary in the laboratory because of the danger of crushing tissue if pressure is applied to the ovary during surgical collection. The Animal Care and Use Committee of West Virginia University approved all procedures for these experiments (ACUC protocol # 06-0401).

Isolation of total RNA

Total RNA was isolated using Tri reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. Briefly, frozen CL tissue was mechanically pulverized while immersed in liquid nitrogen using an RNAase-free porcelain mortar. The pulverized tissue was homogenized in Tri reagent using a glass homogenizer. The RNA was solubilized in RNAase-free-water and its integrity was assessed using 1% agarose gel electrophoresis, all samples were deemed of high quality. Final RNA concentration and purity were determined by spectrophotometry using a NanoDROP 3000 (Nano Drop technologies, Wilmington, DE).

Luteal cell dispersion and purification

For experiment 4, luteal tissue was trimmed to remove surrounding connective tissue, weighed and cut in to small fragments of approximately 1 mm³ size. During these procedures, tissue was immersed in cell dispersion media (CDM, M-199 containing 0.1% BSA, 25 mM HEPES, 100 U/ml fungicide). Tissue fragments were dissociated as previously described [Choudhary E et al, 2005] and luteal endothelial cells were separated according to standard procedures [Choudhary E et al, 2005; Levy N et al, 2001; Mamluk R et al, 1998]. Briefly, magnetic tosylactivated beads (DynaL Biotech, Lake Success, NY) which, specifically attach endothelial cells were added to luteal cell suspension at bead to endothelial cell ratio of 1:3 and endothelial cells were separated. The remaining cell suspension represented an enriched steroidogenic cell population, which also contained other cell types of CL such as fibroblasts, pericytes, immune cells, and possibly few endothelial cells that were not separated by this procedure. Cell viability and density were determined using 4% Trypan Blue exclusion procedure and a hemocytometer; luteal cell viability was usually greater than 95%.

Experiment 1: Microarray -based approach for systematically analyzing the bovine CL transcriptome at two developmental stages: Day-4 and -10, a transition when the luteolytic capability to PGF_{2α} is acquired

Microarray, cDNA labeling, and Hybridization

The microarray assays were performed in Laboratory of Animal Biotechnology and Genomics, Division of Animal and Nutritional Sciences, WVU. Individual RNA samples were used to produce labeled cDNA that was hybridized to the bovine 70-mer long oligo nucleotide probes spotted in duplicates. This array contained 8,329 unique bovine genes developed by Center for Animal Functional Genomics (CAFG) at Michigan State University. Information on the list of genes and their annotations are available via GeneLink database <http://cafg.msu.edu>. Comparisons were made between RNA samples from PGF_{2α} insensitive and -sensitive CL for the changes in gene expression using a total of 6 beef cows (3 replicates x 2 groups). The procedure for cDNA labeling and microarray hybridizations was followed as described previously [Salem M et al, 2006]. Briefly, 30 µg total RNA was reverse transcribed using Superscript II reversetranscriptase (Invitrogen, Hercules, CA) to generate cDNA containing aminoallyl-dUTP. The cDNAs from two experimental groups were labeled randomly with N-hydroxysuccinate-derived Cy3 or Cy5 dyes (GEHealthcare, Piscataway, NJ) to limit the effect of differential dye incorporation. Unincorporated dyes were removed from the cDNA product using a PCR purification kit (Quiagen, Valencia, CA), followed by mixing the Cy3 and Cy5-labeled cDNAs. The labeled cDNAs were concentrated to 20µl using Microcon YM-30 (Millipore, Billerica, MA) and mixed with 130 µl of Slidehyb 3 solution (Ambion, Austin, Texas). The Tecan HS400 automated microarray hybridization station (Tecan US, Durham, NC) was used for array hybridization. The steps in the hybridization were as follows: 1) the slides were subjected to 60°C for 2 min and prehybridized at 55°C for 30 m using prehybridization solution (5 SSC, 1% SDS, 1% BSA) with medium agitation, 2) brief washing was carried out at 60°C for 1 min and 140µl of labeled cDNA mixture was loaded onto the hybridization chamber, 3) hybridization was performed at 60 °C for 3 h followed by 55°C for 13 h, 4) washing was carried out twice with in 2X SSC, 0.1% SDS and twice with in 0.1X SSC, 0.1% SDS at room temperature, and 5) two more washes were given with 0.1X SSC followed by rinsing the slides with water and finally drying by centrifugation.

Microarray Data analysis

After hybridization, the gene array was scanned for spots using ScanArray Lite microarray scanner (Perkin Elmer). The spots were aligned, integrated with original GAL files and signal intensities were quantified using ScanArray Express software (Perkin Elmer, Wellesley, MA). Micro array raw data were processed using GenePix Auto Processor (GPAP) software (<http://darwin.biochem.okstate.edu/gpap3/>) as described previously [Salem M et al, 2006]. Briefly, GPAP utilizes R statistical language, Bioconductor and a LIMMA package to preprocess the raw data by signal filtering, background correction and normalization. The data points with signal intensity values in both channels less than baseline value of 200 were filtered-out and the spots with log-transformed (base 2) ratio outside of 2 standard deviations from the mean were removed as outliers and average of technical replicates within and across replicates were calculated. Raw data were quality controlled manually by flagging and removing any spurious spots from analysis. The preprocessed expression data were normalized by the Loess-global intensity-dependent normalization. The GPAP output value consisted of M value [$\log_2(\text{cy5}/\text{cy3})$], t-statistic, P value (probability), and B-statistics for each spot. Two-fold or more changes in the expressions of genes were indicated by M value ≥ 1.0 (up-regulated) or ≤ -1.0 (down-regulated). The genes with two-fold or more changes in expression with $P < 0.05$ were considered significantly different and selected for further analysis. The microarray data were deposited (according to Microarray Gene Expression Data Society Standards) in NCBI gene expression omnibus (GEO). The curated microarray data can be retrieved with the series accession number GSE10662.

Validation of Microarray Data by Real-time RT-PCR

The pattern of gene expression observed in microarray analysis was confirmed by real-time RT-PCR. Total RNA from CL samples was used for cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). The real-time PCR was performed according to the method described by Pfaffl in 2001 [Pfaffl MW, 2001]. This approach takes into consideration the actual efficiency of each primer. The cDNA generated from pooled RNA samples (D-4 and -10 CL) was diluted serially 10-fold and calibration curve for each gene was generated to determine the efficiency of each primer. The PCR reaction was standardized for optimum

efficiency between 95 to 105% for 9 selected genes. The sequences of each gene primer were designed using primer3 software [Rozen S and Skaletsky H, 2000]. The names, abbreviations, primer sequences and accession numbers for the examined genes are listed in Table 1. The real-time PCR reaction was performed using 25 μ l total reaction containing 2x SYBR Green supermix (BIORAD) and 0.5 μ M primer concentration and 2 μ l of cDNA prepared from 20 μ l reverse transcriptase reaction using 1 μ g of total RNA. The conditions for the real-time PCR reactions were as follows: an initial reverse transcriptase inactivation and Taq polymerase activation step at 94 °C for 3 min, followed by total 40 cycles of 94°C for 30s to denature; 55-60 °C to anneal; and an extension at 72°C for 1 min. The melt-curve was analyzed to make sure genes of interest produced single amplicons. The internal control glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used to normalize the expression values due to differences in amount of RNA. Previous studies from our laboratory and by other investigators have validated the use of GAPDH as a normalizing standard while assessing gene expression at different luteal developmental stages [Buratini J, Jr. et al, 2007; Wright MF et al, 2001]. In the current studies, there were no differences in the values of GAPDH expression from samples collected on day 4 and day 10. The day-4 expression value was set as a calibrator sample (control) and the data were expressed as fold change in day-10 CL mRNA compared to D-4 CL mRNA normalized to GAPDH. The differences in the mean values of mRNA expression between two groups were analyzed by t-test using statistical analysis system JMP 3.0. Gene expression values with a $P < 0.05$ were considered significantly different.

Experiment 2: Responsiveness of selected differentially expressed genes to in vivo treatment with exogenous $PGF_{2\alpha}$.

Real-time RT-PCR. Luteal tissues were collected and RNA samples were isolated for real-time RT-PCR as described above. The selected genes analyzed in this experiment were calcium/calmodulin-dependent protein kinase kinase 2, beta (CAMKK2), protein kinase N1 (PKN1), tyrosine 3monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), regulator of G-protein signaling 2, 24 kDa (RGS2), guanine nucleotide binding protein (G protein), beta polypeptide 1 (GNB1), SPARC-like 1 (hevin) (SPARCL1). The selection of these genes was based on unpublished results from our laboratory and from literature review of genes whose expression was likely to be affected by $PGF_{2\alpha}$ [Choudhary E et

al, 2005; Juengel JL et al, 1998; Sen A et al, 2005]. The sequences and accession numbers of the primers are represented in Table 1.

Experiment 3: Semi-quantitative Western Blotting and Immunohistochemistry -based Approaches for Analyzing CAMKK2 Expression in the Bovine CL During Developmental Transition from Day-4 to -10, and Identifying the Luteal Cellular Source of CaMKK2.

Semi-quantitative Western Blotting. Proteins from frozen luteal tissue were isolated as previously described [Sen A et al, 2004]. Briefly, pulverized frozen tissue was homogenized in homogenization buffer (containing 20 nM Tris-HCL, 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (Eastman Kodak Company, Rochester, NY), 20 (g/ml leupeptin, and 20 mM 2-mercaptoethanol (Gibco BRL, Gaithersburg, MD). The luteal-homogenate was centrifuged at 100 X g for 10 min to separate coarse cellular particles. Protein concentration in the samples was determined by BioRad assay (Hercules, CA) with BSA (Gibco) as standard. Initially, 5, 10, 20, and 40 (g / lane of sample protein were used for semiquantitative western blot analysis as previously described [Sen, 2004 #148]. For assessment of development and treatment effects on amount of CAMKK2 expression, 40 µg / lane of sample protein were used. The following primary antibodies were used in this experiment: mouse anti-actin monoclonal antibody (used at a dilution of 1:3000 [v / v]; Chemicon International, Inc., Temecula, CA); three CAMKK2 polyclonal antibodies were tested: SC 50341, SC 9629 (Santa Cruz Biotechnology, Santa Cruz, CA), and AP7117b (ABGENT, San Diego, CA). The antibodies were used at a dilution of 1:200, 1:200, and 1:50 [v / v] respectively. The following secondary antibodies were used in this experiment: anti-rabbit (1:5000 [v / v]; Amersham Pharmacia Biotech, Piscataway, NJ), anti-goat (1:5000), and antimouse (1:30,000 [v / v]; Gibco) and anti-goat (1:4000 v / v; Gibco) horseradish peroxide-conjugated antibodies.

Validation of the semi-quantitative western blot analysis and stripping conditions has been determined previously [Sen A et al, 2004]. The intensity of the signal corresponding to the protein of interest was standardized by the corresponding intensity of the actin control in that sample. Normalization of data allowed us to estimate, in a semi-quantitative manner, the amounts of protein in the samples of interest.

Immunohistochemistry. Approximately one hour after surgical collection, portions of corpora lutea were fixed for 48 h by immersion in Bouin's solution (75 ml saturated picric acid, 25 ml formaldehyde, 5 ml glacial acetic acid). The tissue was processed by standard histology methodology for embedding tissue in paraffin (Paraplast plus; Tyco Healthcare Group LP, Manfield MA). Ten μm thick sections were prepared from Day-10 bovine CL (n = 3 different cows) using a HM 325 microtome (Fischer Scientific, Pittsburgh PA). Deparaffinized tissue sections were processed for immunohistochemistry according to the manufacturer instructions of a VECTASTIN Universal Quick kit (Vector Laboratories, Inc. Burlingame, CA). Briefly, after quenching endogenous peroxidase activity in 0.3% H₂O₂ in methanol for 30 minutes, sections were incubated in working solution of blocking serum to reduce non-specific binding. Sections were incubated overnight at 4°C with primary antibody, CAMKK2 goat polyclonal IgG (SC9629, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 (vol/vol). The antibodies, SC50341 (Santa Cruz Biotechnology) and AP71176 (ABGENTA) were also tested at a dilution of 1:100 and 1:25 (vol/vol) respectively. Incubation with secondary antibody and streptavidin/peroxidase complex were carried out as indicated by the manufacturer's instructions (Universal Quick kit). Detection was made using the substrate, 3,3'-diaminobenzidine tetrahydrochloride chromogen solution (DAB, prepared according to the manufacturer's instructions; Biomedica Corp. Foster City, CA) at room temperature for 3 min. Slides were counterstained in Harris's Hematoxylin for 30 sec, and serially dehydrated at room temperature in ethanol and finally transferred into xylene for the application of cover slips using a mounting medium (Gel/Mount Biomedica Corp. Foster City, CA). The specificity control for the immunohistological detection was determined by 1) pre-incubation of the primary antibody with excess antigenic peptide (1 μg peptide/1 μg antibody prepared in blocking serum, SC9629 and AP71176), and 2) incubation with normal rabbit or goat serum in lieu of the primary antibody (for the rabbit and the goat primary antibodies). These slides were later observed under an Olympus PROVIS AX70 microscope (Olympus America Inc., Melville, NY) for the presence/absence of specific brown color accumulation indicating immunoreactivity and for microphotography.

Experiment 4: Role of CAMKK2 in mediating the antisteroidogenic actions of increase in $[Ca^{2+}]_i$ stimulated by $PGF_{2\alpha}$

The working hypothesis for this experiment was that the CAMKK2 mediate the progesterone inhibitory actions of increased $[Ca^{2+}]_i$ stimulated by $PGF_{2\alpha}$. Our prediction was that if the activity of CAMMK2 is inhibited, then ability of $PGF_{2\alpha}$ to inhibit the progesterone synthesis is abolished under *in vitro* conditions. We used commercially available STO-609 (Tocris Bioscience, Ellisville, MO) as CAMKK2 inhibitor. STO-609 (7H-Benz [de] benzimidazo[2,1-a]isoquinoline-7-one-3-carboxylic Acid) is utilized as specific inhibitor of CAMKK. It has no significant effect on activities of CaM-KII ($IC_{50} \sim 10 \mu\text{g/ml}$), MLCK, CaM-KI, CaM-KIV, PKA, PKC, and p42 MAP kinase ($IC_{50} > 10 \mu\text{g/ml}$). Indeed, STO-609 is 5-fold more potent in inhibiting CAMKK beta than CAMKK alpha. The working concentration of 10-20 $\mu\text{g/ml}$ of STO-609 was able to completely inhibit the activity of CAMKK beta without significantly affecting the activity of other CaM kinases [Mount PF et al, 2008; Stahmann N et al, 2006; Tokumitsu H et al, 2002]. The enriched steroidogenic cells isolated from Day-10 of non-lactating beef cows were plated in 15 mm 24 -well culture plates at a cell density of 1×10^5 cells/well containing medium M199. Plated cells were treated with 20 $\mu\text{g/ml}$ of STO-609 in presence or absence of $PGF_{2\alpha}$ (1000 ng/ml) and incubated overnight at 37° C in presence of 5% CO_2 . Following day, media was collected and frozen for progesterone estimation by RIA. The RIA used for measurements of progesterone in the culture media has been described previously [Sheffel CE et al, 1982]. The standard curve for this RIA ranged from 10 pg/ml to 800 pg/ml.

Results

Experiment 1: Microarray analysis of the bovine CL transcriptome during the developmental transition from Day-4 to -10.

Our microarray analysis identified 167 genes (~2% of the analyzed transcriptome) that were differentially expressed as (with ≥ 2 fold change in expression; M value ≤ -1 or ≥ 1 ; $P < 0.05$) the bovine CL transitioned from a $PGF_{2\alpha}$ -resistant (D-4) to a $PGF_{2\alpha}$ -sensitive (D-10) stage. Of these 167 genes, the majority was up-regulated in D-10 CL (77%). Gene families that were dynamically up-regulated during this developmental transition most likely reflect a combination of genes involved in the process of active steroid secretion and in acquisition of

sensitivity to $\text{PGF}_{2\alpha}$ -induced luteolysis. The Gene ontology classification of the differentially expressed is shown in Table 2. List of genes with in different categories are listed in Tables 3-11.

One objective of this study was to identify potential genes involved in cell signaling that might participate in acquisition of luteolytic sensitivity to $\text{PGF}_{2\alpha}$. Twenty differentially expressed transcripts were found to be involved in various cell-signaling pathways. The identity, accession numbers, P values and foldchange data of these transcripts are presented in Table 3. Interestingly, nine of these twenty genes are linked to cell-signaling associated with G-protein coupled receptors. For example, a component of the heterotrimeric G-protein complex, guanine nucleotide-binding protein (G protein), beta polypeptide 1 (GNB1) was up-regulated in D-10 CL relative to D-4 CL, as were other genes such as SLIT-ROBO Rho GTPase-activating protein 1 (SRGAP1), Rho GTPase activating protein 8 isoform 1 (ARHGAP8), adenosine A1 receptor (ADORA1), protein kinase N1 isoform 1 (PKN1), and ADP-ribosylation factor 6 (ARF6). Genes in this same category but that were down-regulated in D-10 CL relative to D-4 included: a potent inhibitor of G protein signaling, regulator of G-protein signaling 2, 24 kDa (RGS2); the adrenergic, alpha-1B, receptor (ADRA1B); and a gene encoding the G-protein coupled receptor-98 (GPR98). The transcripts of casein kinase 2, alpha prime polypeptide and NOTCH2 preprotein were up-regulated in D10 CL and these proteins are involved in notch signaling. The mRNAs of two genes mediating Ca^{2+} signaling, such as, calcium/calmodulin-dependent protein kinase kinase 2, beta (CAMKK2) was upregulated in D-10 CL. Additional cell-signaling genes which were down-regulated in D-10 relative to D4 included a calcium-binding protein P22 (CHP); transcripts encoding tyrosine 3monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ); and a gene called unc-51-like kinase 1 (ULK1).

Validation of microarray by real-time RT-PCR

Nine genes that were identified differentially expressed by microarray were selected for quantification of mRNA by real-time RT-PCR to validate the microarray results. The pattern of expression observed by real-time RT-PCR was similar to the patterns observed by the

microarray analysis. The fold changes in expression of genes in D-10 relative to D-4 with each approach are presented side-by-side for comparison in figure 1. All nine genes showed statistically significant differential expression ($P < 0.05$)

Experiment 2: Responsiveness of selected differentially expressed genes to exogenous in vivo $PGF_{2\alpha}$ treatment

There were developmentally related significant differences in expression of all 6 genes examined. Transcripts for CAMKK2, GNB1, SPARC-like 1 and PKN1 were all increased as the CL transitioned from D-4 to D-10; while transcripts for RGS2 and YWHAZ were decreased in this developmental comparison (figure. 2). Furthermore, expression of CAMKK2 and GNB1 were sensitive to exogenous $PGF_{2\alpha}$ in D-10 and D-4 CL samples respectively. Expression of CAMKK2 increased in D-10 CL from 1.16 ± 0.04 with saline to 2.0 ± 0.25 after treatment with $PGF_{2\alpha}$ ($P < 0.05$) without affecting its expression in D-4 CL. In contrast, the expression of GNB1 was increased >10 fold in $PGF_{2\alpha}$ treated D-4 CL compared to its saline D-4 control (D-4 CL saline, 0.16 ± 0.05 ; D-4 CL with $PGF_{2\alpha}$, 1.94 ± 0.43). The expression of GNB1 was not affected by treatment with $PGF_{2\alpha}$ in D-10 CL. There was no significant effect of treatment on the expressions of RGS2, SPARCL1, YWHAZ, and PKN1 within the examined developmental stages.

Experiment 3: Semi-quantitative Western Blotting and Immunohistochemistry -based Approaches for Analyzing CaMKK2 Protein Expression in the Bovine CL During Developmental Transition from Day-4 to -10, and Identifying the Luteal Cellular Source of CaMKK2 Protein.

Semi-quantitative Western Blotting. From the three CAMKK2-specific antibodies tested, only the Santa Cruz antibodies SC 50341 and SC 9629 detected a protein band of approximately 66 kDa (figure 3 and panel A figure 4). However, because the antigenic peptide for SC 50341 is not commercially available, the SC 9629 was selected for further Western blot analysis. The specificity of this 66 kDa protein was demonstrated because its intensity was greatly reduced or abolished by incubating the antibody with its antigenic peptide. The antibody sometimes detected additional faint bands of high molecular weight, but these were not abolished when

the antibody was pre-incubated with its antigenic peptide (figure 3). The 66 kDa molecular weight band identified corresponds closely to the published size band for this kinase [Tokumitsu H et al, 1995]. This 66 kDa protein band was detected at all protein concentrations (5, 10, 20, and 40 μ g) tested in the samples from all D-10 CL (Panel A figure 4). In contrast, it required 40 μ g protein isolated from D-4 CL to obtain a comparable expression signal obtained with 5 μ g of protein isolated from D-10 CL (Panel A, figure 4). As in previous studies from our laboratory, a 43-kDa protein was detected with the antibody for actin. The intensity of this 43-kDa band was similar for each corresponding protein concentration used regardless of luteal development (Panel A, figure 4). The semi-quantitative Western blot analysis using 40 μ g/lane allowed us to estimate the amount of protein corresponding to CAMKK2 expressed in samples prepared from d-4 and -10 CL (Panel B, figure 4). The mean \pm SEM for the actin corrected ratio for CAMKK2 in the D-10 CL was 1.0 ± 0.1 , while for the D-4 CL, it was only 0.57 ± 0.04 ($P < 0.01$) (Panel B figure 3). In contrast, $\text{PGF}_{2\alpha}$ treatment had no effect on CAMKK2 expression in any of the luteal developmental stages examined, D-4 ($0.61 \pm .08$) and D-10 (0.93 ± 0.1) samples (Panel B, figure 4).

Immunohistochemistry.

All three CAMKK2-specific antibodies tested in the immunohistochemistry assay were effective in identifying luteal immunoreactive cells in a consistent manner. Cellular localization of CAMKK2 was routinely examined by immunohistochemistry in at least two areas from three corpora lutea ($n=3$, d-10 CL) using the SC 9699 antibody. Similar results were obtained with the other two antibodies tested. Representative photographs of cells expressing immunoreactivity are displayed in figure 5. Repeating the immunohistochemistry procedure on serial sections from each CL on different areas produced similar results to those displayed in figure 5. Specific CAMKK2 immunoreactivity was clearly detected in large and small luteal steroidogenic cells (figure 5, panels B-D). This immunoreactivity was eliminated when the antibody was pre-incubated with the antigenic peptide prior to immunodetection (figure 5, panel A) and the other control described in materials and methods (data not shown). Endothelial cells were not immunoreactive (figure 5, panel D).

Experiment 4: Role of CAMKK2 in mediating the antisteroidogenic actions of increase in $[Ca^{2+}]_i$ stimulated by $PGF_{2\alpha}$

The values of progesterone were expressed as percent of control because of the variations in its concentrations among animals. Therefore the value of the mean basal progesterone concentration is represented as one. The results are presented in figure 6. Treatment with $PGF_{2\alpha}$ significantly ($P < 0.02$) inhibited the basal accumulation of progesterone (0.78 ± 0.1). Basal amounts of progesterone accumulation in the media were not affected by STO-609 (0.99 ± 0.03). The inhibitory effect of $PGF_{2\alpha}$ on basal progesterone was prevented (1.2 ± 0.06) in presence of STO-609. These observations support our hypothesis that CAMKK2 mediate the antisteroidogenic actions of increased intracellular Ca^{2+} - stimulated by $PGF_{2\alpha}$.

Discussion

The present study identified 167 transcripts that are differentially expressed in the developmental transition when the CL acquires sensitivity to luteolytic actions of $PGF_{2\alpha}$. This represents a significant narrowing-down of the list of potential genes involved in modifying the intracellular signaling utilized by $PGF_{2\alpha}$ on its target cells, in such a way, that the actions of $PGF_{2\alpha}$ on the CL become luteolytic. The gene ontologies of the list of differentially expressed genes in corpora lutea from the early through mid-stage, further narrowed the list by allowing identification of 20 genes that were both differentially expressed and whose function was associated with multiple cell-signaling pathways (Table 2). The results from the second experiment allowed the list of genes whose expression might determine that the actions of $PGF_{2\alpha}$ in the CL become luteolytic to be narrowed further. Expression of both CAMKK2 and GNB1 were sensitive to exogenous $PGF_{2\alpha}$. The combined increase in expression of CAMKK2 due to the developmental transition and the $PGF_{2\alpha}$ treatment might play a critical role in increased luteolytic sensitivity to $PGF_{2\alpha}$. This increase in CAMKK2 occurred at a developmental stage when $PGF_{2\alpha}$ has an increased ability to elicit a rise in $[Ca^{2+}]_i$ compared to Day-4 CL [Choudhary E et al, 2005; Sen A et al, 2005]. CAMKK2 has been reported to mediate the action of increasing intracellular Ca^{2+} via phosphorylation of Ca^{2+} /calmodulin-dependent protein kinases (CAMKs) such as calcium/calmodulin-dependent protein kinases I and IV (CAMK1 and CAMK4). Once phosphorylated, the activity of these kinases is increased

10-20 fold [Haribabu B et al, 1995; Selbert MA et al, 1995]. Furthermore, CAMKs have been shown to activate mitogen-activated protein kinases (MAPKs) such as MAPK1 and MAPK3, formerly known as extracellular signal regulated kinase 1/2 or ERK1/2, in several ligand-stimulated pathways [Illario M et al, 2003; Nguyen A et al, 2004; Rosengart MR et al, 2000]. The significance of this possibility is highlighted when considering that CAMK2 has been shown to mediate the actions of $\text{PGF}_{2\alpha}$ by activating MAPKs in cat iris sphincter smooth muscle [Ansari HR et al, 2001]. Also, MAPKs/ERKs signaling inhibited gonadotropin-stimulated steroidogenesis in rat granulosa-derived cell lines [Seger R et al, 2001]. There is significance in the observation that MAPK1 and 3 mediated the $\text{PGF}_{2\alpha}$ -stimulated expression of prostaglandin-endoperoxidase synthase 2 (PTGS2 or COX2) in neoplastic endometrial epithelial cells, which in turn stimulated the synthesis of $\text{PGF}_{2\alpha}$ in a positive feedback mechanism [Jabbour HN et al, 2005]. Such a positive feedback loop has been suggested to be operational within the CL with luteolytic capacity. MAPKs/ERKs have been involved in the $\text{PGF}_{2\alpha}$ -mediated effects in the bovine and rodent CL. For instance, MAPK/ERKs mediate the $\text{PGF}_{2\alpha}$ -induced apoptosis in the buffalo CL [Yadav VK et al, 2005]; and induction of FOS and JUN mRNA expression by $\text{PGF}_{2\alpha}$ is mediated by protein kinase C-dependent-MAPK/ERK pathway in bovine luteal cells [Chen D et al, 2001]. Furthermore, a calcium/calmodulin-dependent activation of MAPK1 and 3 mediates JUND phosphorylation and induction of *nur77* and *20 α -hsd* genes by $\text{PGF}_{2\alpha}$ in luteinized rat granulosa cells [Stocco CO et al, 2002]. Transcriptional activation of CAMKK2 appears to be regulated by activation of G-protein coupled receptor 54 (GPCR54), acting through Gq/11 family of heterotrimeric G-proteins [Becker JA et al, 2005]. Further, transcription of CAMKK2 was regulated by an increase in intracellular Ca^{2+} [Okuno S et al, 1997]. Given that in the bovine CL, $\text{PGF}_{2\alpha}$ stimulates hydrolysis of phosphatidylinositol 4, 5-bisphosphate and mobilizes intracellular Ca^{2+} [Davis JS et al, 1987b; Wiltbank MC et al, 1991] these mechanisms of transcriptional regulation might be relevant. In support of the importance of CAMKK2 in the acquisition of luteal sensitivity to the luteolytic actions of $\text{PGF}_{2\alpha}$ is the finding that YWHAZ was down-regulated in D-10 CL. This would be in agreement with the report that the protein encoded by YWHAZ inhibits the action of CAMKK2 [Davare MA et al, 2004], because down-regulation would increase the activity of CAMKK2. Therefore, taken together, the observations in these studies that a greater expression of CAMKK2 associated with the D-4 to D-10 CL transition, and the ability of

PGF_{2α} to further stimulate its expression in the D-10 CL, and its role in mediating the antisteroidogenic actions of PGF_{2α} strongly supports the interpretation that CAMKK2 might be involved in acquisition of luteal sensitivity to the luteolytic actions of PGF_{2α}. This interpretation is strongly supported by the finding that there was a parallel increase in the amount of CAMKK2 associated with the D-4 to D-10 CL transition (figure 3). More importantly, ability of PGF_{2α} to inhibit the progesterone synthesis was prevented by CAMKK2 inhibitor (figure 6). Although the *in vivo* effect of exogenous PGF_{2α} increased the mRNA encoding CAMKK2, there was no concomitant increase in the amount of CAMKK2 in D-10 CL. This might be simply due to the fact that it takes longer for the effect of PGF_{2α} to be observed at the protein than at the mRNA level. However it is also possible that despite the changes in mRNA, the protein is not stabilized by exogenous PGF_{2α}. Important to the interpretation that CAMKK2 might be involved in acquisition of luteal sensitivity to the luteolytic actions of PGF_{2α} is the observation that both small and large steroidogenic cells are luteal sources of CAMKK2. These cells are then both target for PGF_{2α} actions, and also express mRNA and protein encoding calcium/calmodulin dependent kinase kinase 2, beta in a developmental manner that agrees with our suggestion that CAMKK2 might be involved in acquisition of luteal sensitivity to the luteolytic actions of PGF_{2α}. Identifying the luteal steroidogenic cells as sources of mRNA and protein encoding Ca²⁺/calmodulin dependent kinase kinase 2, beta is also important from the point of view of designing functional studies to up- and down-regulate the expression of this gene in order to test its function.

The expression of gene encoding the protein kinase C inhibitor, YWHAZ, was down-regulated in the D-10 CL and treatment with PGF_{2α} did not affect its expression either in D-4 or -10 CL [Robinson K et al, 1994; Toker A et al, 1990]. YWHAZ has been shown to inhibit the activity of PKCs by interacting with a cystein-rich C1 domain [Robinson K et al, 1994; Toker A et al, 1990]. Importantly, PKCs mediate the luteolytic actions of PGF_{2α} [Wiltbank MC et al, 1991]. Down-regulation of YWHAZ in the D-10 CL is in agreement with the findings by Juengel et al [Juengel JL et al, 1998] that mRNAs for YWHAZ was greater in D-5 sheep CL compared to D-10 and -15 CL and that PGF_{2α} did not affect its expression. Based on these observations, they suggested that YWHAZ might be involved in the luteolytic resistance in the early ovine CL [Juengel JL et al, 1998]. Similarly, lowered expression of YWHAZ during latter stages of the bovine CL development might increase luteal sensitivity to PGF_{2α}. At the same time, their data

provides an independent validation of the data obtained in experiments 1 and 2 of the current study. Lower expression of GNB1 in the D-4 CL might explain, at least in part, the mechanism responsible for luteal insensitivity to $\text{PGF}_{2\alpha}$ in the early CL. The luteal FP receptors are coupled to heterotrimeric G proteins [Miwa M et al, 1990; Olofsson JI and Leung PC, 1996]. Activation of the linked G protein leads to dissociation of $G\alpha$ and $G\beta\gamma$ subunits from the heterotrimeric complex [Davis JS et al, 1987b; Gilman AG, 1987; Simon MI et al, 1991; Wiltbank MC et al, 1991]. Both $G\alpha$ and $G\beta\gamma$ subunits activate downstream signal transduction mechanisms. It is conceivable that lower expression of GNB1 in the early CL could restrict, in part, full intracellular signaling by FP receptors. Indeed, reduced amounts of GNB1 in D-4 CL might explain the reduced ability of $\text{PGF}_{2\alpha}$ to stimulate a rise in $[\text{Ca}^{2+}]_i$ in D-4 CL [Sen A et al, 2005]. In rabbit CL, PLC activators had luteolytic effects similar to that induced by $\text{PGF}_{2\alpha}$, not only on D-9 and D-13 CL, but also in D-4 CL, in which $\text{PGF}_{2\alpha}$ was completely ineffective [Boiti C et al, 2001]. Boiti et al [Boiti C et al, 2001] suggested that the resistance of early CL might be due to impairment in G-proteins coupled to $\text{PGF}_{2\alpha}$ receptor. Interestingly, exogenous $\text{PGF}_{2\alpha}$ increased the expression of GNB1 in D-4 CL but not in D-10 CL. The magnitude of the increase in GNB1 expression in D-4 CL after exogenous $\text{PGF}_{2\alpha}$ was similar to that seen in D-10 CL; perhaps indicating that expression of this gene was already maximally stimulated during the developmental transition from D-4 to D-10. It has been reported that repeated injections of $\text{PGF}_{2\alpha}$ could regress the bovine CL during its early stage [Sayre BL et al, 2000]; thus increased expression of GNB1 after $\text{PGF}_{2\alpha}$ injection might be one mechanism that makes the early CL acquire luteolytic sensitivity to $\text{PGF}_{2\alpha}$. An additional argument in favor of the significance of differential expression of GNB1 in the mechanism of luteolytic sensitivity to $\text{PGF}_{2\alpha}$ is the report that $G\beta 1\gamma 1$ interacts with RACK1, a scaffold protein that interacts selectively with a specific PKC isozyme, $\text{PKC}\beta 11$ [Dell EJ et al, 2002; Stebbins EG and Mochly-Rosen D, 2001]. Each specific PKC isozymes has been shown to interact with a specific type of RACK protein. The protein kinase C specific isozymes, PRKCB and PRKCE, were differentially up-regulated in D-10 CL, and were activated following treatment with $\text{PGF}_{2\alpha}$ [Sen A et al, 2004]. Therefore, greater amounts of GNB1 in D-10 CL might help to localize the RACK1 to the membrane, followed by recruitment of activated PRKCB to the membrane and initiation of signal transduction. This mechanism might participate in acquisition of luteolytic sensitivity to $\text{PGF}_{2\alpha}$. The developmental down-regulation of RGS2 as the CL transitioned from D-4 to D-10 could

participate in the mechanism of greater luteolytic sensitivity of the bovine CL. RGS proteins attenuate the signaling initiated by G proteins by two mechanisms, one by acting as GTPase-activating proteins (GAPs) [Berman DM et al, 1996; Hepler JR et al, 1997], and also by inhibiting G-protein/effector interaction [Heximer SP et al, 1997]. Studies using in vivo and in vitro methods have demonstrated that RGS2 was a potent inhibitor for G α q signaling [Heximer SP et al, 1999; Ingi T et al, 1998]. The Gq has been shown to activate phospholipase C, beta 1 (PLCB1) leading to downstream signaling such as increases in intracellular calcium and activation of protein kinase C [Berridge MJ and Irvine RF, 1984]. Interestingly, FP receptors are coupled to Gq family of G proteins and activate its downstream signaling pathway, such as increase in intracellular Ca²⁺ and activation of protein kinase C [Abramovitz M et al, 1994; Graves PE et al, 1995; Pierce KL et al, 1999; Watanabe T et al, 1994]. In this regard, the activity of RGS2 was inhibited by protein kinase C in a phosphorylation-dependent manner, thereby potentiating the G-protein stimulated signaling pathway [Cunningham ML et al, 2001]. Therefore decreased expression of RGS2 in D-10 CL might potentiate G-protein signaling, thereby increasing sensitivity of the CL to PGF_{2 α} -induced luteolysis. However, exogenous PGF_{2 α} did not affect the expression of RGS2 either in D-4 or D-10 CL indicating a PGF_{2 α} - independent mechanism of transcriptional regulation for this gene during this developmental transition. The developmental down-regulation of adrenergic, alpha-1B, receptors during the luteal transition from D-4 to D-10 could be an additional mechanism contributing to the lower sensitivity to PGF_{2 α} . In vitro studies utilizing bovine luteal cells have indicated that noradrenaline (NA) stimulated progesterone secretion and reduced the ability of PGF_{2 α} to increase the [Ca²⁺]_i [Skarzynski DJ et al, 2000]. A reduction in the expression of adrenergic receptors would abolish luteo-protective effects of NA, and consequently, would increase the sensitivity of the CL to the luteolytic actions of PGF_{2 α} . In summary, these studies have effectively identified CAMKK2, the protein kinase C inhibitor, YWHAZ, GNB1, and RGS2 as important genes that might play important roles in the acquisition of luteal sensitivity to PGF_{2 α} -induced regression. Manipulating the expression of these genes might prove to be effective strategies for developing more effective estrous synchronization practices in mammals, thereby overcoming the limitation of insensitiveness of early CL to luteolytic actions of PGF_{2 α} . However many other genes, which were differentially expressed in this study might be of relevance in many aspects of luteal physiology, and needs further investigation. Finally, it

should be stressed that the above discussion is based on reported actions of these genes in other systems, and therefore, is speculative in nature.

TABLE 1. List of primers and their sequences

Gene	Forward primer	Reverse primer	Accession No
<i>CAMKK2</i>	TGGAGACGAGTATTGCGACA	CGCCCAACGTAGTCAAACCTT	XM587244
<i>YWHAZ</i>	AACGAAGACCGAAACCTCCT	GGTAGGCCTTCACCTTCTCC	NM1744492
<i>BXLbv68</i>	CGGAGGCTGTGCAGTATTATG	GCACACGATGTAGCGAATGA	XM872283
<i>PTPRR</i>	GAGAGGCGAGGGTCCAAC	AGCTGCGACCTTGTGAGAAT	NM001015662
<i>CL1</i>	ACCTCCTGGGTGATGCAGAT	ATGAAGGCGACAGCTCCA	NM205779
<i>RGS2</i>	CGAGGAGAAGCGAGAGAAGA	CCTCAGGAGAAGGCTTGATG	NM001075596
<i>INPP1</i>	ACCACGTTCAAGTGGGACTC	TTCCCACGTGATACACCAG	NM174364
<i>AURKB</i>	AGCGAACAGCCACGATCA	ACCAGCCGAAGTCAGCAAT	NM183084
<i>GNB1</i>	GACAGGGCAGGGGTCTTG	GTCACTGTGGCGTCCACCT	NM175777
<i>SPARCL1</i>	GACCAAGTTTGTGGCACTGA	GAAGTCCGTACAAGCAGGAA	NM001034302
<i>PKN1</i>	ACTTTGGGAAGGTGCTGCT	ATTGGTCACAGCTGCCAAG	XM585967
<i>GAPDH</i>	AATATCATCCCTGCTTCTACTGG	CATACTTGGCAGGTTTCTCCA	NM001034034

TABLE 2. Classification of differentially expressed genes according to their functions

Gene category	Upregulated	Downregulated	Percentage
Steroidogenesis and metabolism	14	3	10.2
Transcription regulation and DNA biosynthesis	25	6	18.5
Protein biosynthesis and modification	29	2	18.5
Antioxidant property	5	-	3
Extracellular matrix and cytoskeletal proteins	12	4	9.5
Hormonal cell signaling	14	6	12
Protein degradation	9	0	5.3
Miscellaneous	14	14	17
Unknown functions	6	4	6

Percentage = percentage of total differentially expressed genes and includes both up and downregulated genes in the particular gene category

TABLE 3: Genes classified under hormonal cell signaling

Genes	Accession No.	M	P
Casein kinase 2, alpha prime polypeptide (<i>CSNK2A2</i>)	CK769872	1.69	0.03
Inositol polyphosphate-1-phosphatase (<i>INPP1</i>)	CK773631	1.43	0.01
Protein inhibitor of activated STAT, 3 (<i>PIAS3</i>)	CK847890	1.37	0.04
Guanine nucleotide-binding protein (G protein), beta polypeptide 1 (<i>GNB1</i>)	CK775516	1.34	0.05
Regulator of Calcineurin 2 (<i>RCAN2</i>)	CK846982	1.32	0.01
Protein tyrosine phosphatase, receptor type, R (<i>PTPRR</i>)	CK773728	1.26	0.01
Calcium/calmodulin-dependent protein kinase kinase 2, beta (<i>CAMKK2</i>)	CK777876	1.22	0.01
Protein kinase N1 isoform 1 (<i>PKN1</i>)	CK777128	1.3	0.04
SLIT-ROBO Rho GTPase activating protein 1 (<i>SRGAP1</i>)	CK849538	1.12	0.04
Formin binding protein 4 (<i>FNBP4</i>)	CK775025	1.04	0.01
Adenosine A1 receptor (<i>ADORA1</i>)	CK774279	1.12	0.01
ADP-ribosylation factor 6 (<i>ARF6</i>)	CK771198	1.09	0.04
Unc-51-like kinase 1 (<i>ULK1</i>)	CK778217	1.07	0.01
Rho GTPase activating protein 8 isoform 1 (<i>ARHGAP8</i>)	CK847626	1.26	0.03
NOTCH homolog 2 preproprotein (<i>NOTCH2</i>)	CK848127	1.05	0.02
Adrenergic, alpha-1B-, receptor (<i>ADRA1B</i>)	CK771135	-1.54	0.01
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (<i>YWHAZ</i>)	CK849522	-1.06	0.01
Calcium binding protein P22 (<i>CHP</i>)	CK849565	-1.00	0.01
Regulator of G-protein signaling 2, 24 kDa (<i>RGS2</i>)	CK948092	-1.06	0.01
G-protein coupled receptor 98 (<i>GRP98</i>)	CK774105	-1.03	0.01

1M = 2fold change

TABLE 4. Steroidogenesis and Metabolism

Name of the gene	Accession No.	M	P
Aldehyde dehydrogenase 1	CK849144	1.69	0.03
NADH-cytochrome b5 reductase	CK849313	1.48	0.01
Ubiquinol-cytochrome c reductase complex 7.2kDa protein isoform a	CK777947	1.30	0.02
Proline dehydrogenase (oxidase) 1	CK770371	1.35	0.01
Ornithine decarboxylase-like protein	CK776792	1.24	0.03
Pyruvate kinase, liver and RBC (PKLR)	CK778286	1.15	0.02
ATP citrate lyase (ACLY)	CK956511	1.12	0.01
Tricarboxylate transport protein (CTP)	CK773874	1.95	0.01
Glycine dehydrogenase	CK770834	-1.50	0.02
Glutamine-fructose-6-phosphate transaminase	CK776508	-1.15	0.02
Dolichyl-phosphate beta-glucosyltransferase	CK775119	-1.01	0.03
Apolipoprotein A1 (APOA1)	CK943048	1.79	0.01
Fatty acid binding protein (heart)	CK848552	2.30	0.01
Alpha-2-macroglobulin receptor-associated protein precursor	CK770545	1.71	0.01
Mevalonate (diphospho) decarboxylase	CK778011	1.42	0.01
Homolog of yeast long chain polyunsaturated fatty acid elongation	CK943782	1.25	0.01
Lipid phosphate phosphatase-related protein type 2	CK778352	1.24	0.01

TABLE 5. RNA processing, transcription regulation and DNA synthesis

Name of the gene	Accession No.	M	P
Activating transcription factor 4	CK949341	2.02	0.02
Zinc finger protein 652 isoform 2	CK770292	1.83	0.01
Zinc finger protein 287 ((Zinc finger protein SKAT-2)	CK771322	1.88	0.01
Homeodomain interacting protein kinase 4	CK775259	1.46	0.01
LIM homeobox 9	CK772768	1.45	0.01
CDC-like kinase 1	CK848317	1.38	0.01
RNA-binding protein S1	CK773442	1.63	0.01
Ring finger protein 5 isoform 1	CK774044	1.24	0.05
Williams Beuren syndrome chromosome region 22	CK774061	1.47	0.01
Ring finger protein 38 (RNF38)	CK778301	1.18	0.02
Paired box protein Pax-8	CK774334	1.18	0.01
Suppressor of Ty 6 homolog	CK954466	1.23	0.01
Zinc finger protein 197 (ZnF20)	CK776693	1.13	0.01
PHD finger protein 10	CK847333	1.17	0.01
R3H domain containing 1	CK775332	1.13	0.03
E2F family member 8	CK774280	1.03	0.01
DNA-directed RNA polymerase III 47 kDa polypeptide ((RPC4))	CK772296	1.05	0.01
U5 snRNP specific	CK769585	-1.29	0.01
Pirin	CK776403	-1.16	0.01
Nuclear receptor subfamily 1, group H, member 2	CK773128	1.02	0.02
Steroid receptor (TR2-11)	CK769911	-1.27	0.01
G16 (HsRma1) (Ring finger protein 5)	CK775904	1.01	0.01
TAR DNA-binding protein-43 (TDP-43)	CK849045	-1.29	0.02
Early B-cell factor associated zinc finger transcription factor	CK847452	-1.05	0.02
Estrogen receptor-related protein	CK846360	1.03	0.03
RNA binding motif protein 19	CK846624	1.02	0.03
GIY-YIG domain containing 2	CK778699	1.50	0.05
Polymerase (DNA directed), alpha 2 (70kD subunit)	CK774876	1.39	0.01
DNA polymerase delta subunit 3	CK773351	1.28	0.02
Tyrosyl-DNA phosphodiesterase 1	CK775329	-1.03	0.01
C-terminal binding protein 2 (CTBP2)	CK771864	2.10	0.04

TABLE 6. Protein biosynthesis and modification

Name of the gene	Accession No.	M	P
Eukaryotic translation initiation factor 4A, isoform 2	CK846258	1.31	0.01
Activator of 90 kDa heat shock protein ATPase homolog 1 (AHA1)	CK776415	1.24	0.01
Disulfide isomerase	CK776692	1.17	0.02
Eukaryotic translation initiation factor 3	CK849347	1.14	0.01
Elongation factor 1-gamma (EF-1-gamma)	CK773855	1.15	0.01
Elongation factor for selenoprotein translation	CK772036	1.42	0.03
60 ribosomal protein L17	CK771192	1.23	0.02
40S ribosomal protein S27	CK771367	1.23	0.05
40S ribosomal protein S10	CK772050	1.39	0.01
40S ribosomal protein S9	CK772107	1.66	0.01
60S ribosomal protein L31	CK773025	1.98	0.01
40S ribosomal protein S15	CK774452	1.23	0.02
40S ribosomal protein S25	CK775730	1.02	0.02
Ribosomal protein L27	CK776228	1.28	0.01
60S acidic ribosomal protein P2	CK776925	1.35	0.01
40S ribosomal protein S29	CK846156	1.04	0.01
40S ribosomal protein S24	CK847945	2.03	0.02
60S ribosomal protein L26	CK848441	1.32	0.01
60S ribosomal protein L12	CK848743	1.27	0.01
Ribosomal protein, large, P0 (RPLP0)	CK849066	1.14	0.01
60S ribosomal protein L7a	CK849145	1.69	0.01
60S ribosomal protein L9	CK849400	1.14	0.01
40S ribosomal protein S7	CK849595	1.17	0.02
60S ribosomal protein L13	CK849627	1.92	0.01
40S ribosomal protein S5	CK849697	1.60	0.01
40S ribosomal protein S11	CK945135	1.71	0.01
60S ribosomal protein L13	CK946216	1.33	0.01
40S ribosomal protein S29	CK949225	1.28	0.01
40S ribosomal protein S15a	CK953239	1.14	0.01
Heat shock 70kDa protein 5 (grp78)	CK848695	-1.4	0.02
Translation initiation factor IF-2, mitochondrial precursor (IF-2Mt)	CK769193	-1.07	0.02

TABLE 7. Antioxidant property

Name of the gene	Accession No.	M	P
Selenoprotein P-like protein precursor	CK777177	2.05	0.05
Peroxiredoxin 5	CK773035	1.86	0.02
Glutathione S-transferase A2	CK774267	1.67	0.01
Glutathione S-transferase A1	CK778456	1.57	0.01
Glutathione peroxidase 3	CK770347	1.49	0.01

TABLE 8. Extracellular matrix and cytoskeletal proteins

Name of the gene	Accession No.	M	P
SPARC-like 1	CK849369	1.77	0.01
Chondroitin sulfate glucuronyltransferase	CK775983	1.58	0.02
Proteoglycan 1 precursor-like	CK769587	1.47	0.02
Serine protease inhibitor, Kunitz type, 2	CK849325	1.33	0.01
Plakophilin 1- cell to cell adhesion molecule	CK774824	1.31	0.01
Metalloproteinase inhibitor 4 precursor (TIMP-4)	CK849811	1.23	0.01
Putative hyaluronan receptor for endocytosis	CK778012	1.16	0.02
Collagen, type XXII, alpha 1	CK847130	1.29	0.01
Serine/threonine-protein kinase MARK1	CK771345	1.05	0.01
Keratin 7	CK848899	1.23	0.03
Dynactin-1 (150 kDa dynein-associated polypeptide)	CK774327	1.11	0.05
Actin-related protein 6 (hArap6)	CK776995	-1.09	0.02
Keratin 8	CK778284	-1.29	0.03
Type V procollagen alpha 2 chain	CK849288	-1.14	0.02
Plakophilin 2	CK776740	-1.65	0.02
Myosin regulatory light polypeptide 9	CK848506	1.26	0.02

TABLE 9. Miscellaneous

Name of the gene	Accession No.	M	P
Solute carrier family 39 (zinc transporter), member 4	CK776598	1.20	0.03
Lutheran blood group	CK772614	1.38	0.02
SPATA2	CK773652	1.68	0.01
Prostate stromal protein ps20	CK777307	1.23	0.01
SYAP1	CK769966	1.12	0.01
Potassium voltage-gated channel KQT-like protein 2 isoform c	CK772601	1.10	0.01
Secretion regulating guanine nucleotide exchange factor	CK846026	1.04	0.01
Solute carrier family 35, member C2	CK777438	1.06	0.03
Survival motor neuron protein	CK774453	1.10	0.01
Dendritic cell protein GA17	CK848907	1.03	0.05
Ferritin H subunit	CK848958	1.04	0.01
ERIC1	CK940341	1.38	0.05
Transmembrane protein 176A (TMEM176)	CK770329	-1.37	0.01
Macrophage C-type lectin Mincle	CK770579	-1.19	0.02
Aurora kinase B (AURKB)	CK771597	-1.10	0.02
Potassium voltage-gated channel subfamily H member 2	CK773510	-1.71	0.01
Fat cell-specific low molecular weight protein	CK774965	-1.71	0.04
Sialin	CK845898	-1.30	0.02
PKC-interacting cousin of thioredoxin (thioredoxin-like)	CK848430	-1.05	0.01
Sprouty-like protein	CK849801	-1.13	0.02
Chromosome 10 open reading frame 119	CK848711	-2.50	0.01
Synleurin	TC313344	-1.04	0.01
ABC transporter ATP-binding protein	CK845991	-1.19	0.02
Chloride channel 2	TC351877	-1.00	0.05
Nuclear casein kinase and cyclin-dependent kinase substrate 1 (Nucks1)	CK770111	-1.04	0.04
Solute carrier family 35, member F2	CK770565	-1.09	0.05
Phosphoglycerate mutase family member 5 (PGAM5)	CK847884	1.14	0.03
Tumor protein, translationally-controlled 1 (TPT1), P23	CK774391	1.16	0.01

TABLE 10. Protein degradation

Name of the gene	Accession No.	M	P
Cathepsin K preproprotein (CTSK)	CK849357	1.29	0.01
F-box protein 36	CK944568	1.37	0.03
Napsin A preproprotein	CK770924	1.24	0.05
Pleckstrin homology-like domain family A member 1	CK773551	1.24	0.03
Apoptosis-associated speck-like protein containing a CARD (ASC)	CK954618	1.21	0.03
AFG3-like protein 1	CK776738	1.22	0.02
Granzyme M precursor	CK774851	1.16	0.04
UV excision repair protein RAD23 homolog A	CK776179	1.15	0.02
Matrix metalloproteinase 23 (MMP-23)	CK846714	1.02	0.05

TABLE 11. Unknown functions

Accession No.	M	P	Accession No.	M	P
CK777911	1.70	0.01	CK846545	1.08	0.01
CK773556	1.63	0.05	CK770138	1.16	0.04
CK773395	1.61	0.02	CK773493	1.02	0.03
CK773691	1.30	0.02	CK774123	1.49	0.01
CK778328	1.34	0.03	CK775464	1.01	0.03
CK777602	1.33	0.01	CK846118	1.00	0.01
CK848506	1.26	0.02	CK770657	-1.02	0.05
CK845982	1.26	0.04	CK772076	-1.28	0.01
CK774613	1.15	0.03	CK775423	-1.33	0.01
CK769920	1.10	0.02	CK773288	-1.01	0.01

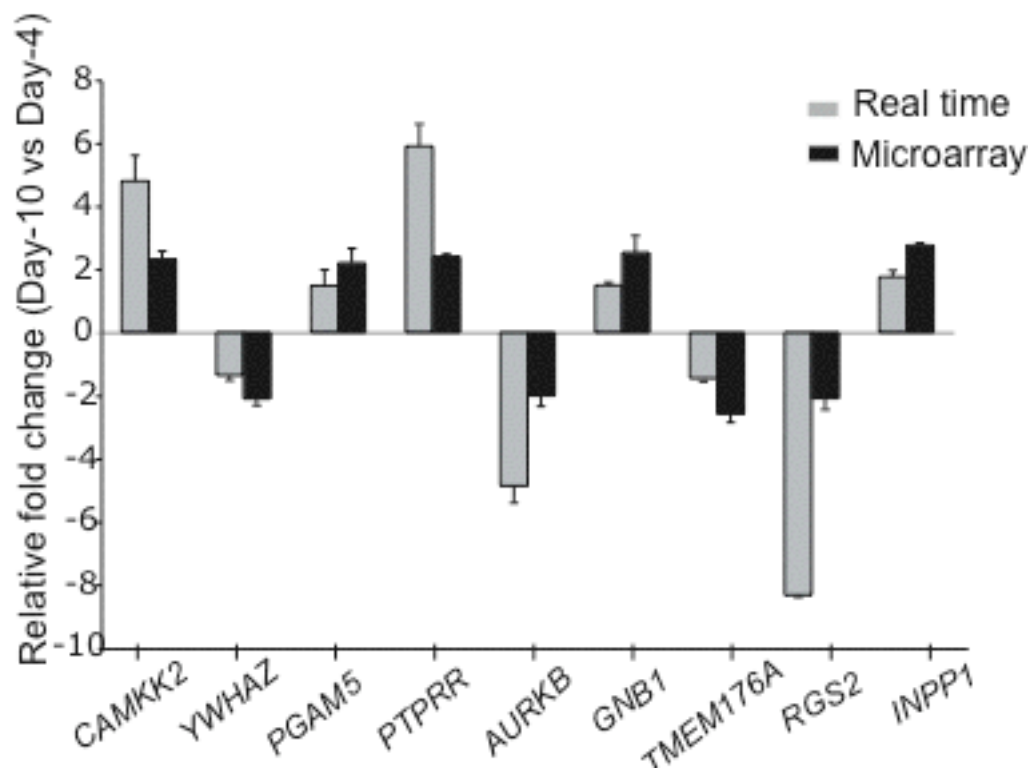


Figure 1. Real-time RT-PCR confirmation of 9 differentially expressed genes in Day-10 CL compared to Day-4 CL that were identified by microarray analysis, $P < 0.05$. CAMKK2: calcium/calmodulin dependent kinase kinase 2, beta; YWHA: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; PGAM5: phosphoglycerate mutase family member 5; PTPRR: protein tyrosine phosphatase, receptor type, R; AURKB: aurora kinase B; GNB1: guanine nucleotide binding protein (G protein), beta polypeptide 1; TMEM176A: transmembrane protein 176A; RGS2: regulator of G protein signaling 2, 24kDa; INPP1: inositol polyphosphate-1 phosphatase. The data is shown as mean \pm SEM, values with differing letters denoting statistically significant differences ($P < 0.05$).

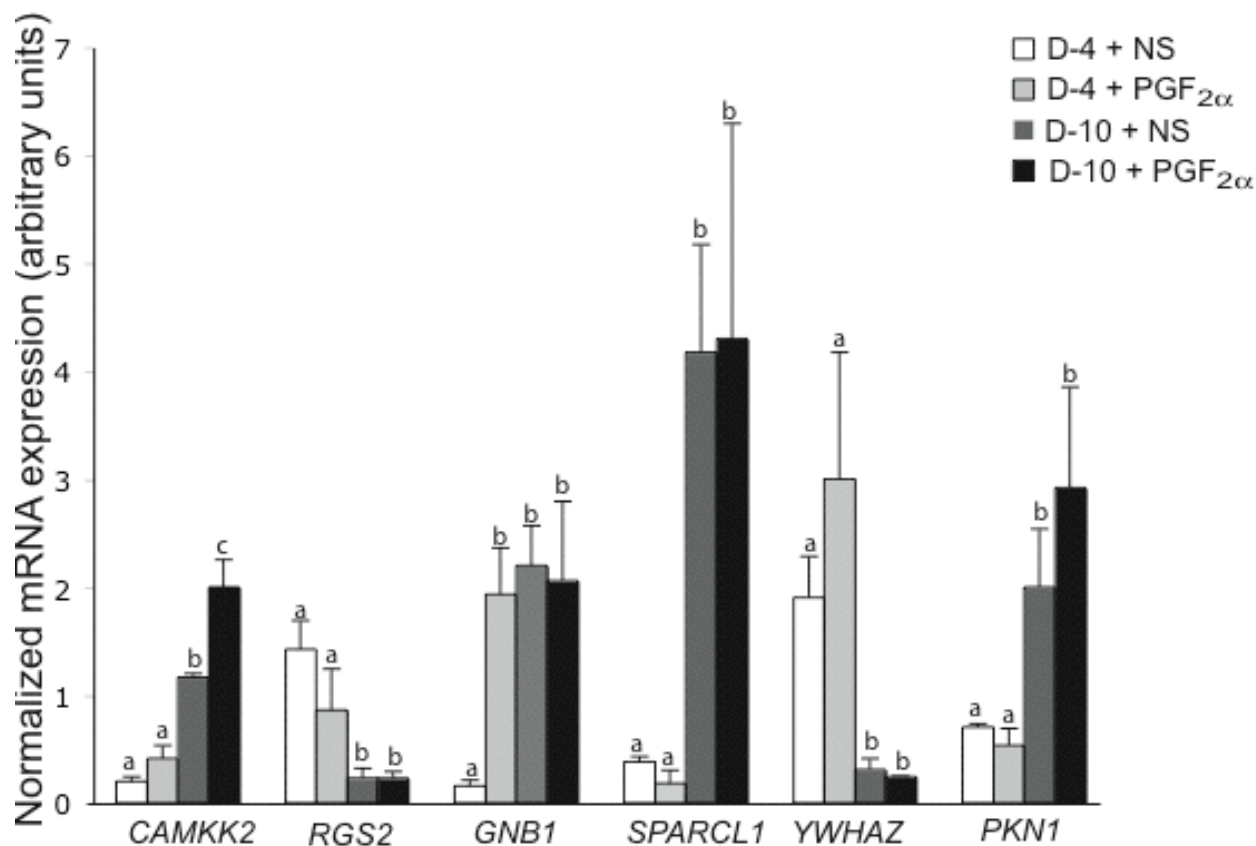


Figure 2. Real-time RT-PCR analysis of the effect of exogenous PGF_{2α} on expression of selected genes that are identified by microarray to be differentially expressed in Day-10 CL compared to Day-CL, $P < 0.05$. NS represents normal saline. CAMKK2: calcium/calmodulin dependent kinase kinase 2, beta; YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; RGS2: regulator of G protein signaling 2, 24kDa; GNB1: guanine nucleotide binding protein (G protein), beta polypeptide 1; SPARCL1: SPARC-like 1; PKN1: protein kinase N1. The data is shown as mean \pm SEM, values with differing letters denoting statistically significant differences ($P < 0.05$).

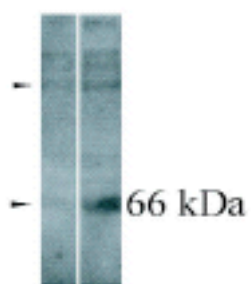


Figure 3. Specificity of the 66 kDa protein band detected by the SC 9629 antibody. In this representative Western blot, 30 μg sample proteins isolated from a day10 corpus luteum was run in each lane. The right lane is a representative Western blot demonstrating that the CaMKK2 antibody detected a protein band of approximately 66 kDa (right panel). The left lane correspond with the experimental conditions where the primary antibody was preincubated with an excess amount of antigenic peptide prior to its use in the Western blot.

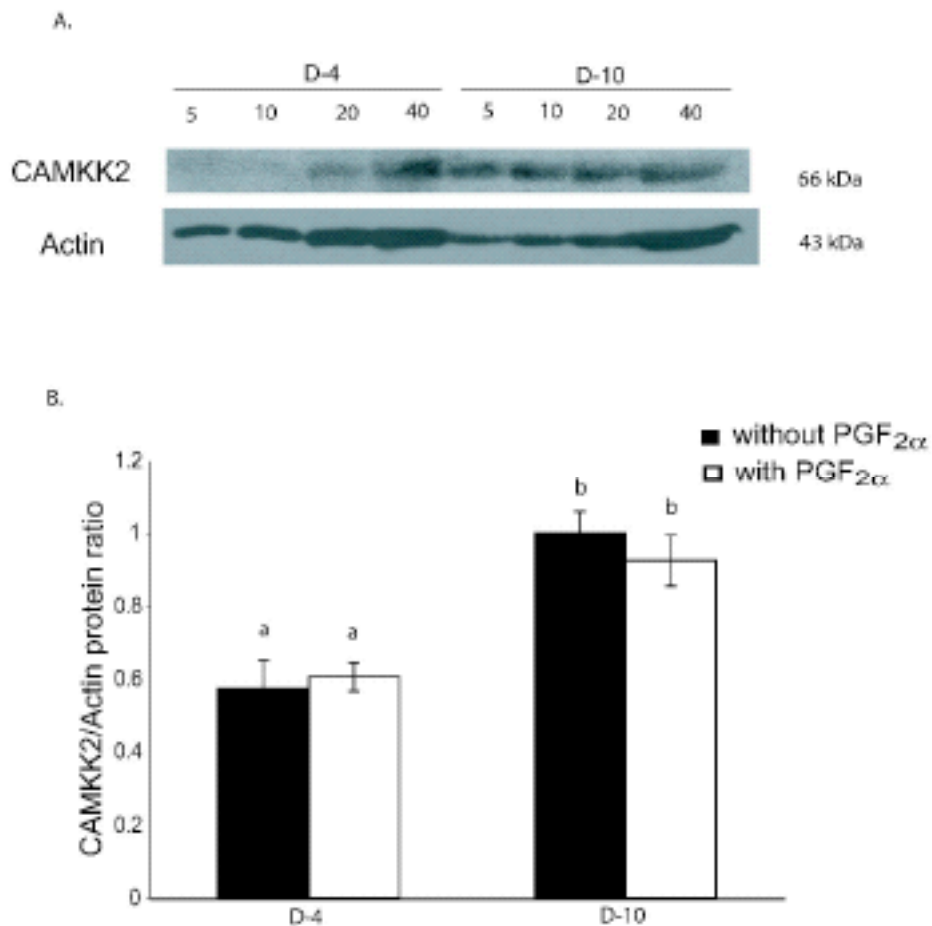


Figure 4. Representative Western blot analysis of the calcium/calmodulin dependent kinase kinase 2, beta (CAMKK2) expressed in the bovine CL and semi-quantitative analysis of the densitometry derived from Western blots using protein samples isolated from d-4 and d-10 bovine corpora lutea. Panel A compares the total sample protein needed to detect CaMKK2 in protein samples isolated from day 4 and 10 corpora lutea. For each developmental stage, 5, 10, 20, and 40 μg / lane sample protein were used. This representative Western blot demonstrates the amount of CaMKK2 and actin expressed in each protein samples collected at d-4 (lanes 1-4) and d-10 (lanes 5-8) of the estrous cycle. The CaMKK2 antibody detected a protein band of approximately 66 kDa, whereas the actin antibody detected a protein band of about 43 kDa. In panel B, the y-axis shows the ratio of the optical density (o.d) for CaMKK2 corrected by the detected o. d. for its corresponding actin. The data is shown as mean \pm SEM, values with differing letters denoting statistically significant differences ($P < 0.001$).

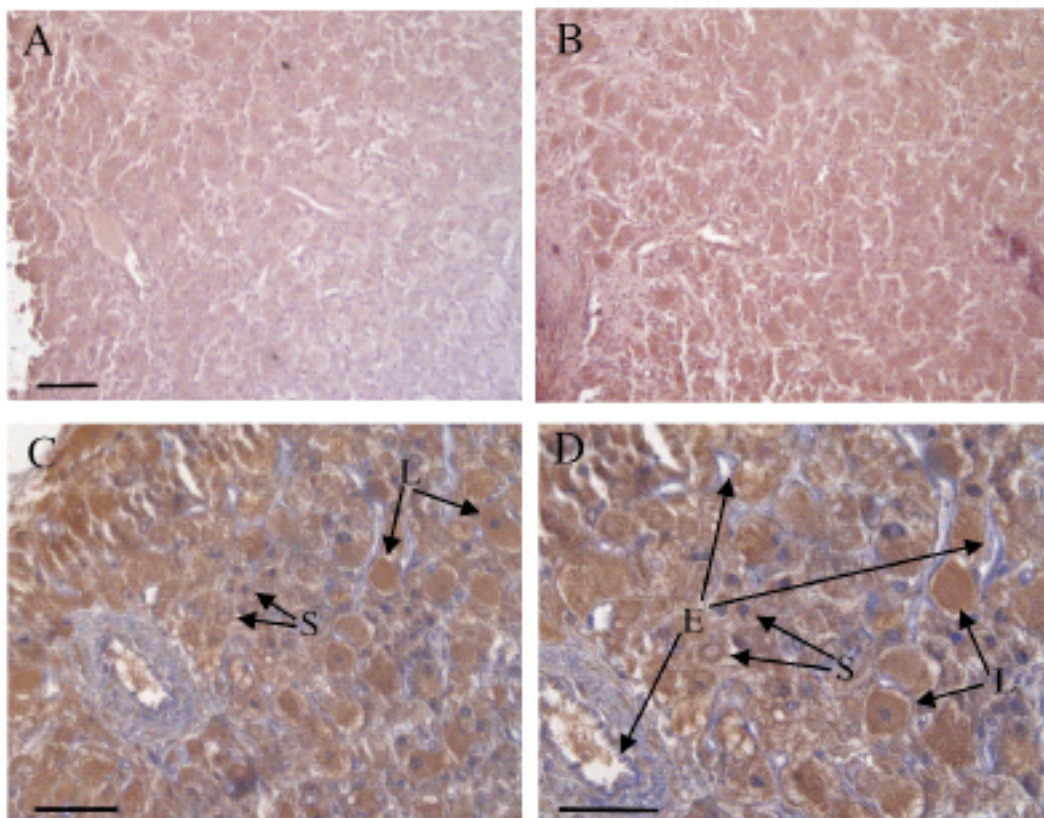


Figure 5. Specific detection of calcium/calmodulin dependent kinase kinase 2, beta (CAMKK2) in day 10 bovine CL (panels A and B) by immunohistochemistry. Panel A demonstrates the specificity of the assay by a dramatic reduction of immunoreactivity when the primary antibody was pre-incubated with the antigenic peptide. Panel C shows immunoreactivity in small (S) and large luteal cells (L). Panel D illustrate lack of immunoreactivity in endothelial cells (arrow labeled E) and immunoreactivity in the cytoplasm of a large (arrow labeled L) and small (arrow labeled (S) luteal cells. Some unidentified cells are observed in the lumen of the blood vessel. Images in panels A and B are shown at the same magnification and the bar on the lower right corner of panel A indicates 100 μm . In panels C and D, the bars in the lower right corners indicate 100 and 50 μm respectively. The nuclei were counterstained by brief exposure to Harris's Hematoxylin solution.

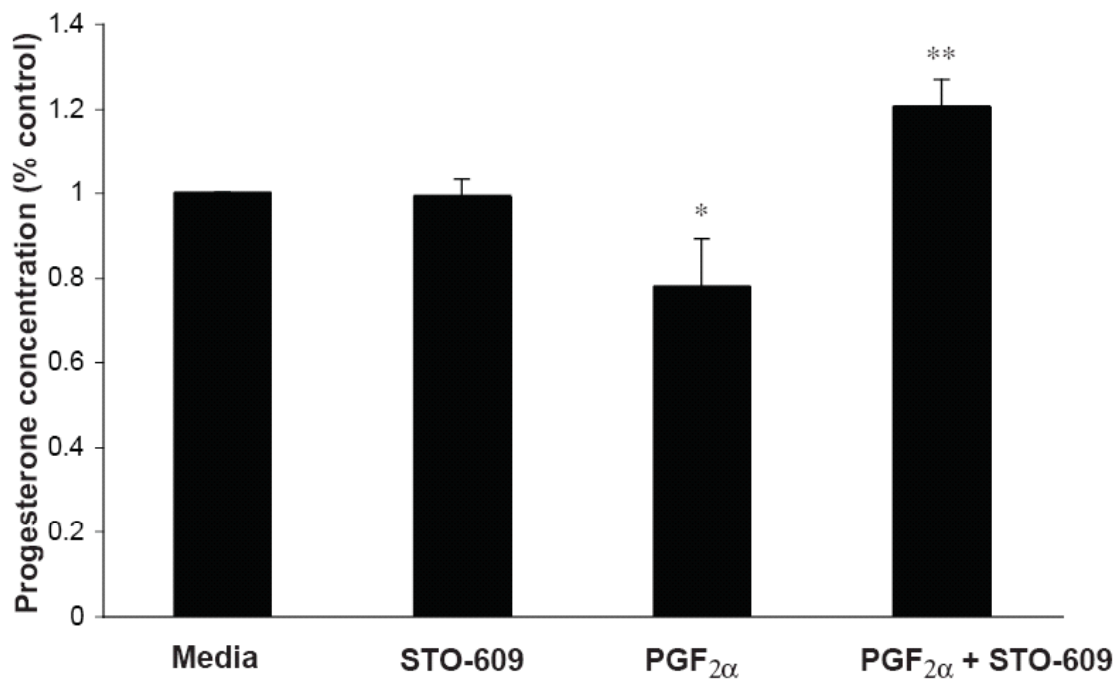


Figure 6. Role of CAMKK2 in mediating the antisteroidogenic actions of PGF_{2α}. This figure represents the accumulation of progesterone in media secreted by steroidogenic cells derived from D-10 CL (n=4) over a period of 12 h. The treatments include media (control), CAMKK2 inhibitor, STO-609 in presence or absence of PGF_{2α}. The progesterone values are expressed as percentage of control (mean ± SE), and the bars with symbol * and ** indicate significant differences (P<0.05) between treatment groups.

Chapter V: Discussion and future studies

The current studies were carried out to investigate the mechanisms responsible for insensitivity of the early developmental stages of the bovine CL to luteolytic actions of $\text{PGF}_{2\alpha}$. Lack of $\text{PGF}_{2\alpha}$ receptors does not seem to explain this differential response because early CL already have high affinity receptors and $\text{PGF}_{2\alpha}$ can elicit distinct physiological response in early CL [Choudhary E et al, 2005; Levy N et al, 2000]. This led to general working hypothesis that differences in post-receptor signaling events activated by $\text{PGF}_{2\alpha}$ in the early versus the mature CL might be responsible for differential responsiveness to luteolytic actions of $\text{PGF}_{2\alpha}$.

Previous study by Sen et al [Sen A et al, 2004] investigated the expression of array of PRKC isozymes in the developing and mature CL. The results of this study showed that bovine CL expressed α , βI , βII , ϵ and μ isozymes. Interestingly, the amount of PRKCE was greater in D-10 CL compared to the D-4 CL and only steroidogenic cells expressed PRKCE. These observations led authors to propose that differential expression of PRKCE could explain the mechanism of early luteal resistance to $\text{PGF}_{2\alpha}$. Therefore, ablation or over-expression of PRKCE in steroidogenic cells might provide useful technique to assess its physiological significance and mechanism in acquisition of luteolytic sensitivity. Therefore, siRNA approach was used to specifically downregulate PRKCE mRNA in steroidogenic cells. This approach has effectively downregulated the amount of PRKCE mRNA by ~75% and protein by ~50% and permitted examination of the specific function of PRKCE. Under the reduced cellular concentrations of PRKCE, the ability of $\text{PGF}_{2\alpha}$ to inhibit LH-stimulated progesterone accumulation was significantly inhibited. This observation strongly supported our hypothesis that low amounts/absence of PRKCE in the early CL might be responsible for inability of $\text{PGF}_{2\alpha}$ to inhibit progesterone synthesis. In general, PRKCs mediated antisteroidogenic actions of $\text{PGF}_{2\alpha}$ [Abayasekara DR et al, 1993; Wiltbank MC et al, 1990; Wiltbank MC et al, 1991], however the role of PRKC specific isozymes has drawn little attention to date. Therefore, our results are direct evidence that PRKCE mediate progesterone inhibitory actions of $\text{PGF}_{2\alpha}$. However, detailed mechanism of action and its cellular targets are not known. Inhibition of activity of PRKCE abolished the ability of $\text{PGF}_{2\alpha}$ to increase $[\text{Ca}^{2+}]_i$, which suggested that PRKCE might regulate amounts of $[\text{Ca}^{2+}]_i$ [Sen A et al, 2005]. However, the exact mechanism by which

PRKCE regulates $[Ca^{2+}]_i$ needs to be investigated. In astrocytes activation of PRKCE increased the expression of mRNAs for voltage- dependent calcium channels (VGCCs). In addition, overexpression of PRKCE increased the density of Ca^{2+} currents [Burgos M et al, 2007]. Therefore, the role of PRKCE in expression of VGCCs or their direct activation by phosphorylation-dependent mechanism needs to be investigated. In addition, cellular co-localization studies of PRKCE with VGCCs might provide insight into its specific target sites of action.

It has been proposed that uterine $PGF_{2\alpha}$ amplifies its luteolytic effect by stimulating its own synthesis from CL [Tsai SJ and Wiltbank MC, 1997]. $PGF_{2\alpha}$ released from CL acts in an autocrine manner to complete the process of luteolysis. In early CL, the inability of $PGF_{2\alpha}$ to induce COX-2 expression, a key enzyme in biosynthesis of $PGF_{2\alpha}$, had been suggested to be cause for early luteal insensitivity [Tsai SJ and Wiltbank MC, 1998](discussed in chapter III). Moreover, the increase in $[Ca^{2+}]_i$ activated PLA2 and subsequently released arachidonic acid [Rosenthal MD et al, 1995]. Therefore, expression of PRKCE and its activation by $PGF_{2\alpha}$ in the late CL might increase $[Ca^{2+}]_i$ sufficient to induce COX-2 expression and probably other important genes involved in the metabolism of $PGF_{2\alpha}$. Supporting this idea, PRKCE induced the expression of COX-2 via Raf1-MEK1/2-p44/42 MAPK pathway in cardiac muscle [Xuan YT et al, 2005]. Therefore, the expression of key PG metabolic genes including COX-2 in PKC ϵ -down regulated cells was investigated. Surprisingly, PRKCE-downregulation did not show significant effects on the expression of any of the genes tested (discussed in Chapter III). However, in the bovine luteal cells, activation of PRKC by $PGF_{2\alpha}$ stimulated Raf/MEK1/MAPK pathway, which was suggested to activate the luteal genes containing AP-1 binding sites [Chen DB et al, 1998]. Therefore, possibility of involvement of other PRKC-specific isozymes in activating this metabolic pathway and subsequent induction of $PGF_{2\alpha}$ -metabolic genes during luteolysis needs to be investigated.

The role of Ca^{2+} in inhibiting and supporting the synthesis of progesterone has been reported, which suggested its biphasic role in steroidogenesis (discussed in chapter III). Our observations strongly suggested that by simply raising the intraluteal Ca^{2+} concentration, one could inhibit

progesterone synthesis irrespective of the developmental stage of CL. However, we did not quantify the amounts of $[Ca^{2+}]_i$ that are required to inhibit progesterone synthesis. Therefore, titrating the quantity of progesterone-inhibitory concentrations of Ca^{2+} might be very important in designing suitable Ca^{2+} -based estrous synchronization protocols. In addition, understanding the $[Ca^{2+}]_i$ homeostasis in CL is important in designing calcium based protocols. It is known that $PGF_{2\alpha}$ stimulates a rise in $[Ca^{2+}]_i$ via release from internal source (ER). However, the function of Ca^{2+} that is derived via VGCCs during $PGF_{2\alpha}$ stimulated anti-steroidogenesis needs further investigation. Recently it has been reported that human luteinized granulosa cells express L- and T-type VGCCs and T-type channel regulate LH-stimulated influx of Ca^{2+} and steroidogenesis [Agoston A et al, 2004]. In addition, $PGF_{2\alpha}$ stimulated influx of extracellular Ca^{2+} via receptor linked Ca^{2+} channels during uterine contraction [Perusquia M and Kubli-Garfias C, 1992]. Importantly, our preliminary results (data not shown) showed that mRNAs for L, N, and T-type VGCCs are expressed in bovine CL. Therefore utilization of specific Ca^{2+} channel blockers during inhibition of LH-stimulated progesterone by $PGF_{2\alpha}$ might provide insight into their specific roles during luteolysis. In addition, studying the expression patterns of VGCCs and IP3 receptors in early and mature CL might help to understand the mechanisms responsible for ability of $PGF_{2\alpha}$ to stimulate greater magnitudes of $[Ca^{2+}]_i$.

It is apparent from our previous results that there were difference in the developmental expression/quantities of certain signal molecules (for example, $PKC\epsilon$ / $[Ca^{2+}]_i$) that might partly explain the difference in responsiveness of CL to $PGF_{2\alpha}$. Supporting this observation, investigators have reported that differential expression of PRKC inhibitors (YWHAZ, PKCI-1), or $PGF_{2\alpha}$ metabolic enzymes (COX-2, PGDH) might be responsible for differential luteal sensitivity (discussed in chapter IV). Therefore, there might be global changes in the expression of genes associated with $PGF_{2\alpha}$ signaling during transition of CL from early to mid-developmental stage. This approach could explore and identify the novel signaling molecules that might play an important role during acquisition of luteal sensitivity. Accordingly, our microarray approach has identified 167 genes that are differentially expressed during transition from D4 to D-10 CL and the gene ontology allowed us to categorize the identified genes into various functional groups. Consistent with our hypothesis, we identified 20 various hormonal cell signaling genes that were developmentally differentially expressed (table 3, chapter IV).

The role of one of these 20 cell-signaling genes, CAMKK2 during acquisition of luteolytic sensitivity to $\text{PGF}_{2\alpha}$ was investigated. The expression of both mRNA and protein of CAMKK2 were up-regulated in the D-10 CL. Importantly, increased CAMKK2 expression occurred at a developmental stage at which $\text{PGF}_{2\alpha}$ can stimulate a greater magnitude of $[\text{Ca}^{2+}]_i$. Therefore, greater availability of CAMKK2 in the mature CL might be important in mediating the actions of rise in $[\text{Ca}^{2+}]_i$ stimulated by $\text{PGF}_{2\alpha}$ and subsequent inhibition of progesterone synthesis. Supporting its role in antisteroidogenesis, both SLC and LLC expressed CAMKK2 and endothelial cells do not appear to express CAMKK2. Importantly, inhibitor of CAMKK2 prevented the ability $\text{PGF}_{2\alpha}$ to inhibit progesterone synthesis, providing direct evidence for involvement of CAMKK2 in antisteroidogenic action of $\text{PGF}_{2\alpha}$. However, the specific intracellular targets and mechanism of action of CAMMK2 needs to be examined. It has been shown that CAMKK2 is the upstream activator of AMPK and activation of AMPK with metformin inhibited progesterone synthesis in bovine granulosa cells [Hurley RL et al, 2005; Tosca L et al, 2007; Woods A et al, 2005]. AMPK inhibits the rate limiting enzymes in fatty acid and cholesterol biosynthesis such as acetylcoenzyme A carboxylase (ACC), fatty acid synthase, and 3-hydroxy-3-methylglutaryl-coenzyme A. Cholesterol is a precursor for the synthesis of progesterone in ovarian cells. Accordingly, activation of AMPK inhibited synthesis of progesterone through mitogen-activated protein kinases (MAPKs) such as extracellular signal regulated kinase 1/2 (ERK1/2) in rat granulosa cells [Tosca L et al, 2005]. Therefore, we propose that AMPK might be a likely target for CAMKK2 during its antisteroidogenic action and needs future attention. However as a preliminary step, we examined the expression of AMPK subunit isoforms in two developmental stages of CL. The results indicated that $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, and $\gamma 3$ AMPK subunits were upregulated in D-10 CL (data not shown). However, the significance of differential expression of AMPK subunits during acquisition of luteolytic sensitivity to $\text{PGF}_{2\alpha}$ needs to be investigated. In addition, the activation of AMPK is due to direct phosphorylation at threonine 172 (Thr^{172}) [Hurley RL et al, 2005]. Therefore, examination of activation of AMPK (phosphorylation studies) by $\text{PGF}_{2\alpha}$ might provide direct evidence for its participation during luteolysis.

Based on the results from our current studies, general model for luteolytic signaling pathway induced by $\text{PGF}_{2\alpha}$ can be proposed as shown in figure 1. Increased expression of PRKCE in Day-10 CL and its activation by $\text{PGF}_{2\alpha}$ results in stimulation of greater magnitude of rise in

$[Ca^{2+}]_i$. Greater availability of CAMKK2 in D-10 CL might be activated by the rise in $[Ca^{2+}]_i$. Subsequently, CAMKK2 might activate its downstream targets such as MAPKs and AMPK to inhibit P4 synthesis. Decline in P4 is followed induction of apoptosis by intracellular Ca^{2+} .

In summary, current observations strongly indicated that differential expression of PRKCE and CAMKK2 might be important factors during acquisition of luteolytic sensitivity to $PGF_{2\alpha}$. In addition, magnitude of $[Ca^{2+}]_i$ stimulated by $PGF_{2\alpha}$ is critical in inhibiting synthesis of progesterone synthesis, which suggested that by altering $[Ca^{2+}]_i$ in CL, one could effectively inhibit progesterone synthesis irrespective of the developmental stage of CL. Studying the physiological roles of intracellular signaling molecules such as GNB1, RGS2, YWHAZ during acquisition of luteolytic sensitivity might help to answer the complexity of intracellular mechanism associated with early luteal resistance to luteolytic actions of $PGF_{2\alpha}$.

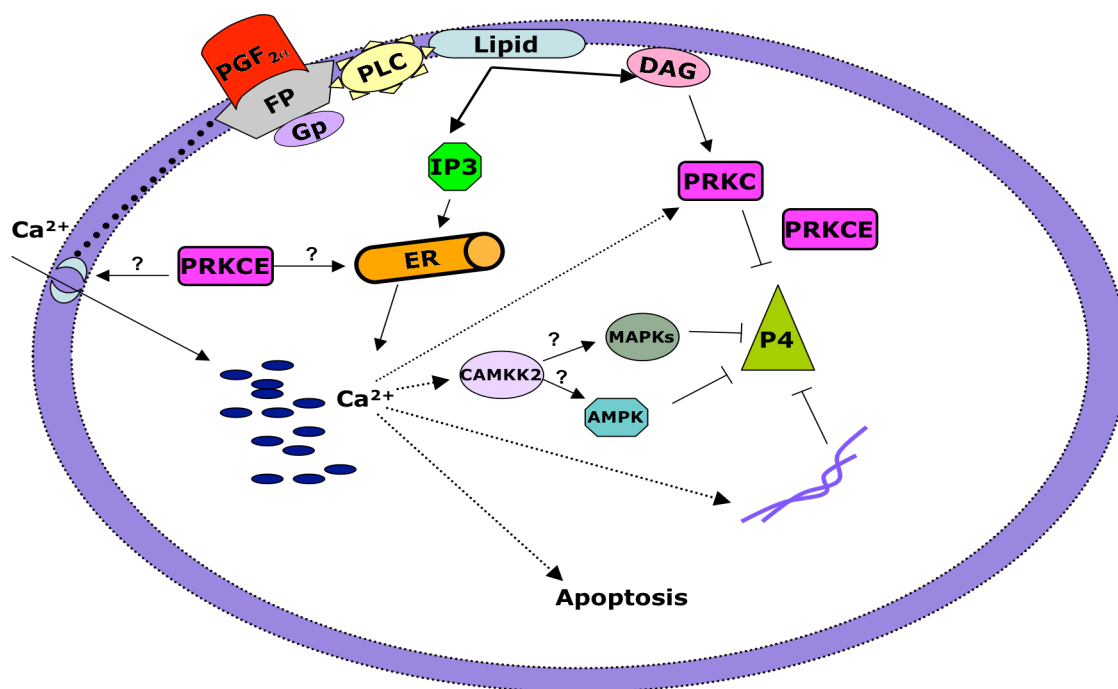


Figure 1. Proposed model for $PGF_{2\alpha}$ -induced luteolytic signaling pathway. PLC: phospholipase C; $PGF_{2\alpha}$: $PGF_{2\alpha}$ receptor; ER: endoplasmic reticulum; PRKC: protein kinase C; PRKCE: protein kinase C epsilon; P4: progesterone; Gp: G-protein; DAG: diacylglycerol; IP3: inositoltriphosphate; CAMKK2: calcium-calmodulin-dependent kinase kinase 2, beta; AMPK: adenosine 5' monophosphate-activated protein kinase; MAPKs: mitogen-activated protein kinases.

Chapter VI: References

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Resume

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EDUCATION

West Virginia University, Morgantown, West Virginia, US

PhD in Biology, 2005 to present, Program: Cell and Molecular Biology program

August 2009

National Dairy Research Institute (NDRI), Haryana, India

Master of Veterinary Science (MVSc), 2004, Area of study: Animal Biochemistry

College of Veterinary Science, University of Agricultural Sciences (UAS), Karnataka, India

Bachelor of Veterinary Science and Animal Husbandry (BVSc & AH) equivalent to DVM, 2001

TECHNICAL EXPERIENCES

- **Animal handling, medicine and some surgical procedures**
- Immunohistochemistry
- Mammalian cell culture and transfection studies
- Microarray
- Small interfering RNA (SiRNA)
- Single Cell Calcium measurements using Epifluorescent Microscopy

- Real-time PCR
- Radioimmunoassay (RIA)
- Western blotting
- Gene cloning and expression
- SDS- polyacrylamide gel electrophoresis and protein purification

AWARDS and HONORS

- Outstanding graduate student award (Doctoral student stipend enhancement fellowship) Department of Biology, West Virginia University, 2008.
- Eberly College of Arts and Science travel award, West Virginia University, 2008.
- Eberly College of Arts and Science travel Award, West Virginia University, 2006.
- Junior Research Institutional Fellowship for Masters Program, Indian Council of Agricultural Research (ICAR), Government of India, 2001-2004.
- Appreciation Certificate from University of Agricultural Sciences, Bangalore, India for excellent performance in All-India combined entrance examination conducted by ICAR, 2001.

PUBLICATIONS

Madhusudan GP, Dev R, Sharma MK, Singh D. Expression of mRNAs encoding tumor necrosis factor-alpha and its receptor-I in buffalo ovary. *Indian J Exp Biol.* 2007; 45(8): 669-75 (PMID: 17877142).

Goravanahally MP, Sen A, Inskeep EK, Flores JA. PKCepsilon and an increase in intracellular calcium concentration are necessary for PGF2alpha to inhibit LH-stimulated progesterone secretion in cultured bovine steroidogenic luteal cells. *Reprod Biol Endocrinol.* 2007; 5: 37 (PMID: 17760987).

Doerr MD, **Goravanahally MP**, Rhinehart JD, Inskeep EK, Flores JA. Effects of Endothelin Receptor Type-A and Type-B Antagonists on Prostaglandin F2alpha-Induced Luteolysis of the Sheep Corpus Luteum. *Biol Reprod.* 2008; 78: 688-696 (PMID: 18077801).

Goravanahally MP, Salem M, Yao J, Inskeep EK, Flores JA. Differential gene expression in the bovine corpus luteum (CL) during transition from early to mid-phase and its potential role in acquisition of luteolytic sensitivity to prostaglandin F₂ Alpha. *Biol Reprod.* 2009; 80 (5): 980-988.

PLATFORM PRESENTATIONS

Goravanahally MP, Sen A, Inskeep EK, Flores JA. Effects of intracellular calcium ion concentration ($[Ca^{2+}]_i$) on luteal progesterone (P₄) accumulation: Implications for developing calcium based protocols to manipulate ovarian cycles. Society for the Study of Reproduction (SSR) 39th Annual meeting 2006, Omaha, Nebraska.

Goravanahally MP, Salem M, Yao J, Inskeep EK, Flores JA. Identification of differential gene expression during transition of bovine corpus luteum from early to mid-phase and their potential role in acquisition of luteolytic sensitivity to prostaglandin F₂ alpha. 2008 Joint ADSA-ASAS meeting 2008, Indianapolis, Indiana.

POSTER PRESENTATIONS

Flores JA, Doerr M, **Goravanahally MP**, Rhinhardt J, Inskeep EK. Endothelin-independent and -dependent luteolytic effects of prostaglandin F₂ alpha in ewes. Society for the Study of Reproduction (SSR) 40th Annual meeting 2008, San Antonio, Texas.

Flores JA, Sen A, **Goravanahally MP** & Inskeep EK. Knockdown of protein kinase C ϵ (PKC ϵ) in bovine luteal steroidogenic cells mitigates the effectiveness of prostaglandin F₂ α (PGF₂ α) in reducing LH-stimulated accumulation of progesterone (P₄). 7th International Ruminant Reproduction Symposium. August 2006, Wellington, New Zealand

Flores JA, Perea FP, Mankey JE, **Goravanahally MP**, Inskeep EA. Effects of Chronic Administration of Endothelin Receptor Type A Antagonist (BQ-610) on Functional Lifespan of the Corpus Luteum in Sheep. July 2009, Society for the Study of Reproduction (SSR) 41st Annual meeting, Pittsburgh, Pennsylvania.

Mankey JE, Perea FP **Goravanahally MP**, Flores JA, Inskeep EA. Effects of Atosiban on Progesterone Production During Early and Mid Cycle in the Ewe. July 2009, Society for the Study of Reproduction (SSR) 41st Annual meeting, Pittsburgh, Pennsylvania

MEMBERSHIP

American Society of Animal Science (ASAS)

Society for Study Reproduction (SSR)

COMPUTER SKILLS

MS office, Adobe Photoshop, Adobe illustrator, statistical software (JMP 3.0)

TEACHING EXPERIENCE

- Contract teacher, Department of Veterinary Biochemistry, College of Veterinary Science, Bangalore, India (2005)
General Biochemistry and Metabolism (Lecture and laboratory)
Clinical Biochemistry (Lecture and laboratory)

- Graduate Teaching Assistant, West Virginia University (2006-2009)
Vertebrate Histology (Biol 441)
Introductory physiology (Biol 117)
The Living Cell (Biol 219)
Introductory Biology (Biol 103)
Introductory Physiology (Biol 115)