# AN ABSTRACT OF THE THESIS OF

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Title: Possible Autocrine/Paracrine Action of Progesterone in the *Ovine* Corpus Luteum

Abstract approved:

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Fredrick Stormshak

The objective of the present research was to determine, by use of the progesterone receptor antagonist RU-486, whether progesterone by autocrine/paracrine action suppresses phosphatidylinositol hydrolysis, and thus, the production of prostaglandin $F_2\alpha$ during the midluteal phase of the estrous cycle in ewes. Two experiments were performed. Experiment 1 consisted of 10 ewes (day 8 of the estrous cycle) with an initial *in vivo* treatment of 10 μg RU-486 in saline or vehicle and blood samples collected before treatment and 10 min after for progesterone analysis. Treatment of RU-486 or vehicle was injected into the ovarian artery and corpora lutea were removed after 10 min of exposure. Fifty mg tissue slices were incubated with treatments of $^3$H myo-inositol (10 μCi) for 90 min followed by incubation (15 min) in the absence and presence of $PGF_2\alpha$ (10 nM), to evaluate the effects of RU-486 and $PGF_2\alpha$ on phosphoinositide hydrolysis. Total labeled inositol phosphates were recovered by use of column chromatography. Data were expressed as cpm of $^3$H myo-inositol/mg tissue. Analysis of variance revealed a significant $PGF_2\alpha$ x RU-486 interaction ($p < 0.05$) arising as a result of both agents being effective in stimulating incorporation of $^3$H myo-inositol into the inositol phosphates. Serum concentrations of progesterone were significantly reduced in both the control and RU-486 treated ewes ($p<0.05$). In a subsequent experiment, luteal tissue was collected on day 8 of the

estrous cycle as in experiment 1. Fifty mg luteal slices were incubated with $^{3}H$ myo-inositol (10 μCi) for 90 min and samples were again exposed to RU-486 (2 μM) in the absence and presence $PGF_{2}\alpha$ (1 μM) for 15 min. Total labeled inositol phosphates were again recovered by use of column chromatography. Comparable to data of experiment 1, *in vitro* exposure of tissue to both RU-486 and $PGF_{2}\alpha$ in experiment 2 caused an increase in incorporation of $^{3}H$ myo-inositol into phosphoinositide phosphates (RU-486 x $PGF_{2}\alpha$ interaction, $p<0.05$). Progesterone levels of the incubation medium demonstrated an increase in response of luteal tissue to RU-486 and was significantly increased by exposure to $PGF_{2}\alpha$ in just 15 min ($p<0.05$). Collectively, these data suggest that progesterone may act nongenomically in an autocrine/paracrine manner to inhibit phosphatidylinositol hydrolysis, and by this action prevent production of $PGF_{2}\alpha$. Such inhibition of endogenous $PGF_{2}\alpha$ production, by progesterone would prevent self-destruction of the CL during the midluteal phase of the cycle.

# Possible Autocrine/Paracrine Action of Progesterone in the *Ovine* Corpus Luteum

By

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Mary Anne Smallman, Author

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# LITERATURE REVIEW

The corpus luteum is an endocrine gland that develops in the ovary following ovulation in all mammalian species. It is an accepted fact that reproduction of mammalian species can be attributed, at least in part, to progesterone synthesized and secreted by the corpus luteum. Interference with the ability of the corpus luteum to produce progesterone, either by natural causes or pharmacologically, results in infertility regardless of whether desired or not. This endocrine gland has been the subject of study for over 50 years primarily from the standpoint of developing methods of contraception in humans and synchronization of estrus in domestic livestock. While much has been learned about the biochemicals that regulate the formation, maintenance and regression of the corpus luteum, the intracellular pathways by which these agents act on these stages of luteal life span are not completely understood.

This thesis will describe research that has been conducted to determine how the corpus luteum arises from the ovarian follicle following ovulation and the morphology of the various cell types that comprise the structure of this mature endocrine gland. Known endogenous factors and their mechanisms of action regulating the synthesis of progesterone and the peptide hormone oxytocin and that ultimately promote regression of the corpus luteum will be examined. Lastly, new concepts will be advanced relative to the possibility that progesterone itself may be an

autocrine regulator of luteal cell function. Results of two experiments utilizing the *ovine* corpus luteum are presented in support of these new concepts.

**The Estrous Cycle**

The estrous cycle begins after sexual maturity in non-primate mammalian species. This particular reproductive cycle differs from the menstrual cycle, which occurs in primates, because menses does not occur. Mammals with an estrous cycle also display a period of sexual receptivity, or standing estrus, shortly before ovulation. What the estrous cycle and menstrual cycle have in common, is their dependency on the release of gonadotropins from the pituitary to promote folliculogenesis and ovulation, the essential hormones that regulate production of ovarian estradiol ($E_2$) and progesterone ($P_4$), and the duration of the cycle.

The ovarian cycle in mammals is regulated by several hormones allowing the necessary physiological changes throughout the estrous cycle to take place. Gonadotropin Releasing Hormone (GnRH) from the hypothalamus acts on the gonadotropes in the pituitary to release Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). Luteinizing Hormone induces ovulation and FSH can bind to their receptors on granulosa and theca cells in the ovary to stimulate the production of estradiol. Luteinizing Hormone is also required to stimulate the production of progesterone after ovulation. Progesterone is essential for the implantation of the blastocyst and a successful pregnancy to occur.

Ovarian cycles consist of a follicular phase and a luteal phase, continuously fluctuating between producing estradiol and progesterone throughout recurring cycles. The follicular phase, or folliculogenesis consists of a growing ovarian follicle and the luteal phase involves development of the endocrine gland, the corpus luteum, which synthesizes progesterone essential for maintenance of pregnancy. Both folliculogenesis, and luteal maintenance, are controlled by the anterior pituitary gland (Messinis et al., 2009) because of their contribution of gonadotropins.

The follicular phase and the luteal phase can both be further broken down into four separate stages to describe the ovarian cycle; proestrus, estrus, metestrus, and diestrus. First, the follicular phase constitutes proestrus, the rise of estradiol from the maturing follicle. Second, once an adequate level of estrogen has been reached, non-primate mammals exhibit a behavioral estrus, with an ovulation taking place soon after. Third, metestrus describes the transition after ovulation in the luteal phase, when luteal cells are transformed from the walls of the ovulated follicle. Lastly, diestrus describes the luteal phase when the corpus luteum is functioning at its highest steroidogenic potential to produce the dominant hormone progesterone.

Granulosa cells are the cell layer lining the antrum of the antral follicle, and during the early follicular phase, they only contain receptors for FSH. Theca interna cells are a layer of flattened spindle-shaped cells outside of the basement membrane of an antral follicle and are endowed with receptors only for LH. These two populations of cells in the ovary work in synchrony to produce the steroid hormone,

estradiol, described with the accepted "2-cell, 2-Gonadotropin Theory" (Hillier, 1994). This current theory is used to describe how granulosa and theca cells during the follicular stage are able to produce estradiol. After binding of LH to its receptor in theca cells, a cascade of events is activated, resulting in the synthesis of testosterone, from its substrate cholesterol. Testosterone can then diffuse out of the theca interna cell and into the granulosa cell. Follicle Stimulating Hormone receptors in the granulosa cells, when bound by FSH, aromatize testosterone converting it to estradiol (Senger, 2012).

*Duration: Species Differences*

The duration of the estrous cycle and the time of ovulation after the onset of estrus, varies by specie. The primary function of the corpus luteum (CL) is to produce progesterone during the luteal phase of the estrous cycle. During pregnancy, the placenta will take over the production of progesterone in some species, the cow (6-8 months), ewe (50 days), and mare (70 days). Seasonality can also be a variable, dependent on specie.

The ewe is a short day seasonal breeder cycling throughout fall and early winter. Their estrous cycle is 16-17 days in length. Standing estrus occurs on day 0 and ovulation takes place 17-18 hours after the onset of estrus. The estrous cycle in the cow is not limited to day-length and occurs every 20-21 days, with an ovulation ranging 6-24 hours after the onset of estrus. The sows' estrous cycle is also non-seasonal reoccurring every 19-21 days with an ovulation occurring on day 2 of estrus.

The mare is a seasonal breeder, but unlike the ewe, the mare cycles during long periods of daylight every 20-22 days. Rats and mice exhibit estrus every 4-5 days with each stage of the estrous cycle lasting just one day. These animals ovulate approximately 8-10 hours after the onset of estrus (Schillo, 2009).

The primate lacks an estrous cycle and instead has a menstrual cycle. The follicular phase of the menstrual cycle is much longer in comparison to the estrous cycle (Cole and Cupps, 1969; Baird et al., 1975). Previous reports have shown that human sperm can only survive up to three days and must be present in the reproductive tract during this phase of the cycle in order for conception to occur (Barrett and Marshall, 1969). However, Bilian et al. (2010) studied conception rates of 851 married Chinese women and determined that the fertile period can actually last up to 7 days prior to ovulation.

*Follicular Development throughout the Cycle*

Techniques used to determine the pattern of growth of preovulatory follicles in cows, initially had conflicting beliefs, largely due to the development of techniques available at the time [Reviewed by Fortune et al. (1988)]. First, basic methods were used, using gross anatomy to count follicles on ovaries at the time of slaughter, with or without measuring steroids in follicular fluid. Eventually, techniques included the use of real-time ultrasonography that could detect large follicles in the ovary. These differences in techniques caused original investigators to disagree whether or not

follicular development was continuous, such as a wave, or if they were independent of the cycle.

The follicular wave was originally defined by Rajokoski (1960) as the change in the number of follicles among the days of the estrous cycle. Rajokoski hypothesized that a two-wave development of follicles existed, and this hypothesis would last twenty years. Eventually the two-wave hypothesis was challenged by Marion et al. (1968) after investigating over 700 ovaries in dairy cows, suggesting that follicles over 10 mm in diameter developed independently of the stage of the estrous cycle and were continuous and constant throughout. Donaldson and Hansel (1968) also presented evidence against waves of follicular growth. But when studies using India ink (dyes) were incorporated, scientists observed that the growth and regression pattern of larger follicles were more rapid towards the end of the cycle (Dufour et al., 1972) shown by large follicles on day 3, regressing by day 8 of the cycle. Similarly, a second dominant follicle visible on day 13 was replaced by day 18, while the ovulating follicle was only identifiable 48 hours before estrus (Hansel and Convey, 1983) disputing Rajokoski's two-wave phenomenon (Ireland et al., 2000).

The conflicting theory that follicular development was a well-controlled phenomenon was solidified with the use of ultrasonography (Fortune et al., 1988; Ginther, 1989). This technique was a less invasive procedure, with the ability to follow the same follicle over time, but still had disadvantages of accurately detecting

follicles greater than 4 mm. This new technology would support the hypothesis of Ireland and Roche (1983), utilizing blood and follicular fluid steroid assays, determining that not only was follicular development controlled by the cycle, but that three successive phases existed, with one selected follicle becoming dominant, either ovulating or becoming atretic, depending on the concentration of progesterone during its dominant phase. Sirois and Fortune (1988) supported this theory after using real-time ultrasonography detecting that seven out of ten heifers showed three waves of follicular development per cycle, with one large follicle becoming dominant per wave, while other heifers had two or even four waves. It was later determined that the variance between the number of follicular waves depended upon several factors, such as dietary intake (Murphy et al., 1991) parity, and lactational status (Lucy et al., 1992) in cows (Ginther et al., 1996).

Research by Evans et al. (2000) contribute to the hierarchy of the dominant follicle theory by use of radiolabeling estradiol in the follicular fluid. Their contribution describes the hierarchy of the dominant follicle, not only in size but also steroid production. Results from this study indicate that dominant follicles produce more estradiol and less progesterone than subordinate follicles.

*Ovulation*

First biochemical, and then physiological events are required for ovulation to occur in mammalian ovarian cycles. Historically (the 1950s), fundamental discoveries were made to understand the mechanisms controlling the estrous cycle

and ovulation (Hansel and Convey, 1983; Spicer and Echternkamp, 1986) such as the development of understanding how hormones bind to their receptors, and the discovery that the hypothalamus and brain control the anterior pituitary. It has since been determined that ovulatory follicles not only grow in size, but the number of LH receptors in both theca and granulosa cells become more responsive to LH increasing their ability to secrete estradiol. First, the preovulatory surge of LH is needed before an ovulation can occur, as well as sufficient levels of estradiol in follicular fluid. Ovulation occurs when the Graafian follicle ruptures, releasing the egg for fertilization (Karsch et al., 1980).

**The Corpus Luteum**

The CL is an endocrine gland or yellow body that is formed from granulosa and theca cells after ovulation of the Graafian follicle in the ovary. Its function is to synthesize progesterone which acts on the uterus to maintain pregnancy and is essential for embryo survival of all mammalian species. In addition, progesterone acts in the hypothalamic-hypophyseal axis to suppress the release of gonadotropins. The steroid hormone progesterone is the major secretory product produced by the corpus luteum, but it is also produced by the placenta and the adrenal cortex. The CL can also synthesize and secrete peptide hormones such as oxytocin, relaxin, and inhibin-activin. The significance of the CL as a source of oxytocin in regulating the duration of the estrous cycles of the mammalian female will be discussed below.

*Origin of Luteal Cells*

In both pregnant and non-pregnant animals, cells in the corpus luteum differentiate from the ovulated ovarian follicle. It is believed that large luteal cells (LLC) developed from granulosa cells after ovulation, and small luteal cells (SLC) originate from theca cells. These cells have since been referred to as granulosa-lutein cells, or theca-lutein cells (Niswender et al., 1985; 2000). Results of early experiments describing the origin of the luteal cell theory were at one time contradictory. Meidan et al. (1990) demonstrated that *bovine* granulosa and theca cells can be stimulated *in vitro* by exposing them to forskolin plus insulin. Their research examined these cells' ability to transform into small and large luteal cells similar to the corpus luteum derived cells, by confirming their similar characteristics. The research by Alila and Hansel (1984) contributed to our understanding of the current theory of luteal cell size by using monoclonal antibodies for granulosa or theca cells. Their research provided evidence suggesting that small and large luteal cells originate from theca and granulosa cells, respectively. In addition, their research also showed that small luteal cells may transform into large luteal cells as the luteal phase progresses, as determined by theca antibody binding to large luteal cells during the mid-cycle (Weber et al., 1987).

*Morphology of Large and Small Luteal Cells*

Morphological characteristics differ between small and large luteal cells. Large luteal cells are also called Type II or D cells and in most species are

approximately 22-35 µm in diameter (Martinez-Zaguilan et al., 1994; Niswender et al., 1994). Large cells have aspherical nuclei (Diaz et al., 2002), and their cytoplasm contains secretory granules. The LLC possesses a plasma membrane that is highly convoluted (Alila and Hansel, 1984). Large luteal cells contain densely packed spherical mitochondria (necessary to produce progesterone) (Fields et al., 1985). They also have rough ER with extensive Golgi and secretory granules, meeting the requirements of an endocrine cell. Lipid droplets (cellular organelles) are found in the cytoplasm, in the LLC (Fields et al., 1992). In the ewe, the total volume and number of these lipid droplets are greater in LLC (Khanthusaeng et al., 2016). Lipid droplets represent an excess of cholesterol esters which are precursors of steroidogenesis (Hawkins et al., 1995).

The small luteal cells, or Type I or I cells, are 12-20 µm in diameter and have spherical nuclei. The cytoplasm of the SLC lack secretory granules. Small luteal cells have very few microvilli on their cell's surfaces and have lipid droplets from which cholesterol esters are transported to the mitochondria for the ultimate production of progesterone (Fields et al., 1985).

In the CL of the sheep, LLC make up 25% of the volume density and SLC make up 17% (Rodgers et al., 1984). However, the number of SLC are 3-5 times greater in the *ovine* CL (O'Shea et al., 1979; Fitz et al., 1982; Rodgers et al., 1983) and four times greater in the *bovine* (Weber et al., 1987).

The production of progesterone varies between small and large luteal cells and among species. In the ewe, LLC produce 10-30 times more progesterone (Fitz et al., 1982; Diaz et al., 2002). While in the cow, LLC produce 7 times more progesterone than small cells (Weber et al., 1987).

Nonparenchymal cells or nonsteroidogenic cells of the CL include the vascular cells; endothelial cells, pericytes, fibroblasts, and immune cells. These make up the biggest population of cells found in the *bovine* CL (Niswender et al., 2000; Slomczyñska and Tabarowski, 2001; Walusimbi and Pate, 2013; Khanthusaeng et al., 2016). Interactions between endocrine producing luteal cells and endothelial cells may indirectly affect steroidogenesis, resulting in endothelial cells playing a role in stimulating progesterone production in both small and large luteal cells. This was shown by a reduced production of progesterone, when luteal steroidogenic cells were isolated from non-steroidogenic cells (Girsh et al., 1995).

This vast population of endothelial cells have a variety of morphological differences, even within the same organ, and have been characterized by five distinct subtypes in the *bovine* CL (Fields and Fields, 1996; Slomczyñska and Tabarowski, 2001; Davis et al., 2003; Khanthusaeng et al., 2016). These endothelial cells are believed to support the luteal cell throughout angiogenesis in the mature CL and may also play a role in both luteolytic and luteotropic events.

*Luteotropins: LH vs Prolactin*

Research conducted during the 1930's examined the role of the anterior pituitary (AP) in regulating ovarian function. Firor (1933) found that hypophysectomy of rabbits 35 min after mating (with an autopsy 24-48 hours later) prevented the formation of new ovulation sites. If hypophysectomy was delayed 50 min post-mating, with autopsy 72-96 hrs later, CL were markedly reduced in size compared to that of the controls. These experiments were among the first to provide evidence that the AP was essential for ovulation and maintenance and growth of the corpus luteum. Further investigation of the AP identified it as the source of the gonadotropins FSH, LH and prolactin (Prl). Foster et al. (1937) managed to give doses of crude preparations of FSH and LH alone, and in combination, to hypophysectomized rabbits immediately after an induced ovulation. FSH alone did not result in the stimulation of luteal tissue formation. However, LH alone or together with FSH, did affect formation of the CL. LH was thought to not only stimulate the development of luteal cells but also secretion of a "corpus luteum hormone."

Early observations by Astwood (1941) revealed Prl as part of the luteotropic complex in the rat. These findings led to further studies utilizing domestic animals to better understand the luteotropin involved in the formation and maintenance of the CL. Whether or not, LH or Prl was the primary luteotropin in ruminants was addressed throughout the 1960's. Determining the role of LH and Prl and their

involvement was at first highly controversial. Denamur and Mauleon (1963) reported that treatment of hysterectomized-hypophysectomized ewes after ovulation with daily injections of prolactin caused CL to be maintained for 20 days. These data were subsequently confirmed by the experiments of Thibault (1966) and thus lead to the conclusion by these investigators that prolactin was luteotropic.

Kaltenbach et al. (1968a) conducted experiments to determine whether the pituitary played a role in regulating CL function. Hypophysectomy of ewes on day 1 of the estrous cycle resulted in either failure of the CL to form or a significant reduction in weight and progesterone content of partially formed CL. The results of these studies only confirmed that the necessary luteotropins for the normal functioning corpus luteum of the estrous cycle originates from the anterior pituitary. Subsequently, Kaltenbach et al. (1968b) went on to show that crude pituitary extracts are luteotropic, when continuously infused in hypophysectomized cyclic or pregnant ewes, resulting in maintenance of the CL.

Schroff et al. (1971) also measured peripheral levels of progesterone, LH, and Prl, detected on days 11-20 in hypophysectomized ewes. It was shown that infusions of crude LH maintained the CL until day 20 in 4 out of 9 animals while pure LH maintained the CL in 5 out of 10 animals. LH and FSH in combination did not maintain CL in any of 8 animals.

Karsch et al. (1971a) using synchronized ewes demonstrated that infusing LH, Prl, or a control vehicle from day 12 of the cycle, until day 20, caused regression of the CL in control and Prl treated ewes, but not those infused with LH. These results suggest that of the two potential luteotropins secreted by the AP, LH appears to be the primary luteotropin.

Karsch et al. (1971b) confirmed that LH from the pituitary could extend the life span of the CL in normally cycling ewes. A continuous venous infusion of purified *ovine* LH beginning 10-12 days after the onset of estrus, maintained the CL up to day 30. The weights and progesterone concentrations of the maintained CL were similar to those of control CL on days 9 to 13 of the cycle, proving that the maintained CL were endocrinologically comparable. These data provide evidence that the CL requires continual support from luteotropic substances to remain functional in normally cycling, intact ewes.

Donaldson and Hansel (1965) reported evidence that LH was the primary luteotropin in the cow shown by extending the life span of the CL with injections of pituitary extracts and *bovine* LH on day 16 of the estrous cycle. Other treatments consisting of *bovine* LH first incubated in 6 M urea (for 1 day at 40°C), inactivated the luteotropic effects of LH and CL regressed similar to those of the control group. These results indicate that pituitary extracts and LH are luteotropic in the cow, just as in the ewe.

*Synthesis of Progesterone*

Research described in the previous section established that LH when administered to the ewe and cow could prolong the life span of the corpus luteum. Whether LH was maintaining structure as well as function of the corpus luteum was not known. However, Mason et al. (1962) were the first to demonstrate that LH when added to cultures of *bovine* luteal tissue stimulated progesterone synthesis. Likewise, Armstrong and Black (1966) recovered CL from cows at various stages of the estrous cycle and found that addition of LH to incubation medium containing luteal tissue stimulated synthesis of progesterone. Cook et al. (1967) examined progesterone synthesis by *porcine* corpora lutea collected during various stages of the estrous cycle and early pregnancy. Luteal tissue was incubated in the presence of LH, FSH or Prl and response to these hormones was measured by incorporation of $^{14}$C-acetate into progesterone and 20α-hydroxy-4-pregnene-3-one. Progesterone synthesis in response to LH was found to be maximal by CL mid-cycle (day 10) and early pregnancy (day 22). Addition of FSH and Prl were without effect in stimulating progesterone synthesis. How LH could stimulate the luteal cell to synthesize progesterone began to become clear by the discovery of LH receptors on the target cells. Diekman et al. (1978) identified the presence of LH receptors in the *ovine* corpus luteum. They were able to quantify the population of receptors present in luteal tissue at various stages of the estrous cycle, and also found that they were greatest on day 10 of the estrous cycle and during early pregnancy. Fitz et al. (1982) showed evidence for LH receptors residing primarily on small luteal cells, but was later discovered that small

and large luteal cells each have an equal distribution of LH receptors (Harrison et al., 1987).

Hoyer et al. (1983; 1984) studied the intracellular signaling pathway LH activates when bound to its receptor in the SLC. It is now accepted that LH, binds to a G-protein coupled receptor (GPCR) in the CL causing activation of adenylate cyclase which converts ATP into 3'-5'-cyclic adenosine monophosphate (cAMP) which then binds to protein kinase A (PKA) that promotes activation of gene expression and the production of progesterone. Segaloff (1990) also confirmed that when LH bound to its receptors in the CL, cAMP was generated. However, large and small luteal cells, when unstimulated by LH or db cAMP have varied responses. The regulation of steroidogenesis has been shown to be different in the small vs large luteal cell, as reviewed by Niswender (2002). Under normal conditions, small cells secrete low levels of progesterone and possess receptors for LH. In contrast, LLC secrete high levels of progesterone and were found to also have receptors for LH (Hoyer and Niswender, 1985); however, they are unresponsive to LH or db cAMP to produce progesterone. Unlike the SLC, steroidogenesis in the LLC was determined to be unregulated, or regulated by something other than cAMP (Hoyer et al., 1983; Niswender, 2002; Mishra and Palai, 2014). To test this response further, Hoyer and Niswender (1985) incubated LLC with cholesterol, ram serum, or 25-hydroxycholesterol, again showing failure to respond to any substrate. Diaz et al. (2002) showed that LLC produce large quantities of progesterone independently of

LH stimulation, suggesting that the PKA pathway may be tonically active in LLCs resulting in a slow continuous release of progesterone. In conclusion, production of progesterone by LLC remains a mystery.

The change in the capacity for steroidogenesis by the CL is an important aspect of luteal physiology. The first challenge this steroid producing cell faces is the ability to obtain the necessary precursor molecule, cholesterol. Luteal cell steroidogenesis requires both low (LDL) and high (HDL) density lipoproteins, as these lipid droplets consist of cholesterol esters, and are supplied to the CL through the direct contact of capillaries for the rapid production of progesterone (Stocco et al., 2007). The number of lipid droplets in the LLC is greater than in the SLC, and is capable of utilizing more cholesterol esters, likely the reason for its greater steroidogenic capacity. Low density lipoproteins are taken up by luteal cells via a receptor-mediated endocytosis mechanism (Diekman and Niswender, 1978; Ahmed et al., 1981) and are found in both small and large luteal cells (Rajapaksha et al., 1997). High density lipoproteins are more selective because of their greater density and requires a carrier protein, the scavenger receptor class B type I (SR-B1) protein which allows the selective uptake of cholesterol esters carried by HDLs in the blood (Rajapaksha et al., 1997). Lastly, the production of cholesterol can also be provided in situations where the cell is deprived of lipoproteins, via the intracellular synthesis of cholesterol esters from acetate (Strauss III et al., 1981). Cholesterol is then

transported from pools of cholesterol esters to the mitochondria, where it is then converted into pregnenolone.

Cholesterol enters the mitochondria by a steroidogenic acute regulatory protein (StAR), first identified by Stocco et al. (1988) using a MA-10 mouse Leydig cell tumor line (Clark et al., 1994). StAR was found to localize to the mitochondria where it assists cholesterol movement from between the outer to the inner mitochondrial membrane, where important P450 enzymes are located. The P450 side chain cleavage (P450scc) enzyme converts cholesterol to pregnenolone (C-21) by hydroxylation and cleaving the carbon chain (C-22-C-27). Pregnenolone exits the mitochondria and enters the endoplasmic reticulum where it is converted into progesterone, by 3β-hydroxysteroid dehydrogenase (3β-HSD) (Mishra and Palai, 2014). This step by P450scc is necessary because hydrophobic cholesterol cannot transverse the aqueous environment and the intermembrane space of the mitochondria. Therefore, two limiting steps have been discovered to be key factors in the production of progesterone, 3β-HSD and the StAR protein (Stocco, 2001).

*Production of Peptide Hormones*

Water soluble peptide hormones are synthesized in the endoplasmic reticulum, transferred to the Golgi apparatus, and packaged into vesicles that can be exported out of the cell. These hormones target receptors on the surface of endocrine responsive cells, which initiate 2$^{nd}$ messengers to bring about a genomic response.

The posterior and anterior lobes of the pituitary gland both secrete peptide hormones via the release of various hypophysiotropic, or releasing hormones, carried out in portal vessels linking the hypothalamus to the pituitary. Releasing hormones can either stimulate or inhibit peptide hormone synthesis dependent on the needs of distant organs. Six peptide hormones are synthesized and stored in the anterior pituitary; ACTH, TSH, FSH, LH, GH and Prl. While two peptide hormones, vasopressin and oxytocin (OT) are only stored in the posterior pituitary, after being synthesized in the magnocellular neurons in the hypothalamus.

Peptidal hormones also appear to be secreted from luteal cells, likely involved in the paracrine regulatory effects in the CL, reviewed by Niswender and Nett (1994). Oxytocin has been characterized to be synthesized in luteal tissue directly, based on data generated by both RIA (Wathes and Swann, 1982; Swann et al., 1984) and immunocytochemistry, where the staining of luteal cells on days 4, 10, and 16 showed OT to be immunoreactive in large luteal cells only, beginning on day 4 and persisting up until day 15 (Sawyer, 1986).

## Luteolysis

### *Uterine Origin and Local Action*

In the mid 1950's, Wiltbank and Casida (1956) were the first to report that hysterectomy of the ewe and cow during the estrous cycle prolonged the life span of the corpus luteum. This research suggested that somehow the uterus was involved in regulating the function of the CL by producing the signal involved with luteolysis.

Subsequent studies in swine (Spies et al., 1958), hamsters (Caldwell and Wright, 1967), and guinea pigs (Poyser, 1976), confirmed these findings that the luteolytic substance is of uterine/endometrial origin. In contrast, in the primate (Burford and Diddle, 1936) and human (Jones and TeLinde, 1941) removal of the uterus has no effect on the life span of the CL.

Rowson and Moor (1966) studied the functional relationship between the conceptus and the CL in sheep by embryo transfer, showing that embryos present on day 12 in otherwise cycling ewes, prolonged luteal life span. Further experiments were conducted using unilateral hysterectomy showing that the maintenance of the CL during early pregnancy was due to release of a presumed "luteotropin" through the venoarterial pathway, allowing passage from the gravid uterine horn to the CL in the ipsilateral ovary.

*Discovery of Prostaglandin $F_{2}\alpha$*

Phariss and Wyngarden (1969) were the first to show that $PGF_{2}\alpha$ injected into pseudopregnant rats terminated the CL. Subsequently, McCracken (1970) showed this in ewes by infusing $PGF_{2}\alpha$ into the arterial supply to ovaries autotransplanted via vascular anastomoses to the neck resulting in the concomitant regression of the CL and reduced progesterone synthesis. Prostaglandin $F_{2}\alpha$ was identified to be released from the uterine vein into the ovarian artery during luteolysis in the sheep (McCracken et al., 1972), rat (Pharriss and Wyngarden, 1969) and cow (Hixon and Hansel, 1974). Mapeltoft et al. (1976) demonstrated that the exchange of $PGF_{2}\alpha$ was

carried through the uterine vein into the ovarian artery, by use of transposed ovarian arteries, reviewed by Ginther et al (1981). Shutt et al. (1975) measured $PGF_2\alpha$ in the CL of women by RIA showing an abundance of $PGF_2\alpha$ present in luteal tissue and higher levels of metabolites for $PGF_2\alpha$ in ovarian venous blood at the time of luteolysis (Shutt et al., 1976). Prostaglandin $F_2\alpha$ is now well known to be the main luteolysin in many species, specifically those dependent on the uterus to control the life span of the CL, such as in the ruminant, rat, and guinea pig. Prostaglandin $F_2\alpha$'s short half-life was demonstrated by Piper et al. (1970) who found that $PGF_2\alpha$ introduced into the general circulation was degraded after one pass through the lungs in the hamster. McCracken (1971) showed that this phenomenon was also true in the ewe using radiolabeled $PGF_2\alpha$ to detect a 99% deterioration rate.

Administration of $PGF_2\alpha$ in the cow (Hafs et al., 1974) was shown to shorten the life span of the CL if given after the first 5-6 days of the cycle (Deaver et al., 1986), and on day 12 of the cycle or later in the pig (Guthrie and Polge, 1976). In the ewe, corpora lutea are resistant to $PGF_2\alpha$ (luteolysis) during the first few days of the estrous cycle, reviewed by Inskeep (1973) and more recently described by Rubianes et al (2003).

Both structural and functional luteolysis have been described, and at first, were highly controversial due to species differences. An example is luteolysis in the mouse, where loss of luteal function occurs well before structural regression,

allowing up to 3 generations of CLs to persist simultaneously (Long and Evans, 1922). This is compared to the situation in the non-pregnant ewe (Thorburn, 1972) where complete spontaneous regression of the CL occurs on days 15-18 of the estrous cycle. Structural luteolysis describes the reduction of tissue and proceeding cell death in the corpus luteum, while functional luteolysis describes the regression and loss of steroidogenic capacity in luteal cells, as reviewed by Pate (1994).

To date, there have been several theories postulating how $PGF_2\alpha$ might be involved in regulating luteolysis. Niswender et al. (1975) reasoned that $PGF_2\alpha$ treatment reduced blood flow to the ovary resulting in apoptosis and cell death. A reduction in LH receptors by $PGF_2\alpha$ was also proposed by Behrman et al (1978). Diekman et al. (1978b) studied this phenomenon using corpora lutea from ewes injected with $PGF_2\alpha$ on day 9 of the estrous cycle. Jugular blood samples were collected at 20 min intervals one hour before the injection and 0.5, 1 or 2 hours after the injection. Depending on treatment group, CLs were removed at various time points after injection (2.5, 7.5, and 22.5 hr), further incubated with $PGF_2\alpha$, and receptors for LH were analyzed. Whether or not active vs inactive LH receptors were involved in controlling luteolysis and the luteolytic action of $PGF_2\alpha$ resulting in the decline of progesterone was examined. Luteal weights and progesterone concentration were recorded. Results indicate that luteal progesterone concentration after the administration of $PGF_2\alpha$ decreased well before a reduction in the number of occupied or unoccupied receptors for LH. These data show that $PGF_2\alpha$ itself is

responsible for the change in progesterone concentration in the CL, and that luteolysis does not require a withdrawal of LH support or a decrease in LH receptors.

Evrard et al. (1978) and Fletcher and Niswender (1982) showed that uncoupling the LH receptor from adenylate cyclase might have been involved by incubating luteal tissue *in vitro* providing evidence that the presence of $PGF_2\alpha$ was necessary for at least 1-2 hours before it would affect progesterone levels. Uncoupling LH receptors from adenylate cyclase, the influx of calcium, and activation of cytotoxic cascades have all been shown to contribute to the luteolytic effects of $PGF_2\alpha$.

*Synthesis of Prostaglandin $F_2\alpha$*

The biochemical pathways, upon which activation leads to the production of $PGF_2\alpha$ were studied over several decades. Hokin and Hokin (1955) first examined the rate of lipid membrane turn-over, which was later defined as phosphatidylinositol hydrolysis. Activation of phosphatidylinositol hydrolysis is a key first step in generating an increase in $PGF_2\alpha$ production by tissues. Not only is $PGF_2\alpha$ a product of phosphatidylinositol (PI) hydrolysis but $PGF_2\alpha$ can also activate the PI hydrolysis in ovarian cells in the rat (Leung, 1985; Leung et al., 1986), cow (Davis et al., 1987; 1988) and sheep (Wiltbank et al., 1989b). Techniques labeling phosphoinositides with either $^{32}P$ or $^{3}H$-inositol were used to quantify to what extent $PGF_2\alpha$ induced phosphoinositide turn-over (Huckle and Conn, 1987; Lapetina and Siess, 1987) and by the late 1980's, the role of phosphatidylinositol hydrolysis (second messenger

system) involved in luteal cell function was intensely studied, as reviewed by Berridge (1987) and Wiltbank (1991). This research paved the way for the new field of phosphoinositide metabolism and the discovery of the PKC second messenger system (Stocco and Kilgore, 1988).

Prostaglandin $F_2\alpha$ binds to a GPCR in the plasma membrane, stimulating the effector enzyme phospholipase C (PLC) cleaving phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to produce two second messengers, diacylglycerol (DAG) and inositol trisphosphate ($IP_3$). Inositol trisphosphate is then released into the cytosol which can further act on the endoplasmic reticulum to provoke a release of intracellular calcium into the cytoplasm. This increase of intracellular calcium activates protein kinase C (PKC), an enzyme which goes on to activate several other pathways, ending with gene transcription or translation involved in mechanisms

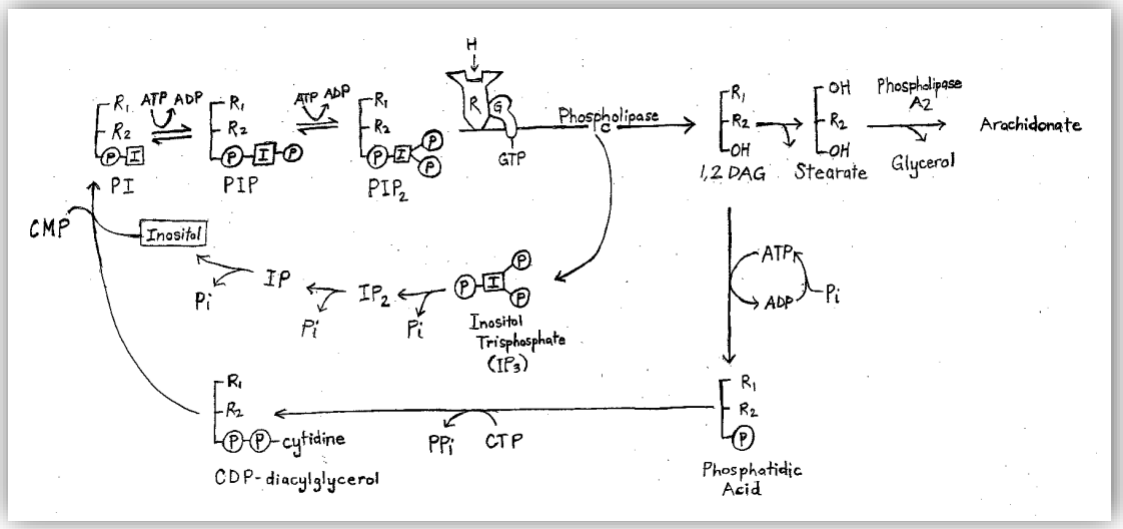


Fig 1. Phosphatidylinositol Hydrolysis (Hokin and Hokin, 1955).

controlling cell growth and metabolism, and the synthesis of OT, (to be discussed in more detail in the subsequent section).

Prostaglandin $F_{2}\alpha$ is a lipid molecule and a member of the eicosanoid family, consisting of a 20-carbon, polyunsaturated fatty acid arising from arachidonic acid (AA) stored in membrane phospholipids. Arachidonic acid is the primary precursor for all prostaglandins (PGs). Phospholipase $A_2$ ($PLA_2$) cleaves AA arising from DAG, while the cyclooxygenases (COX 1 and 2) converts AA into prostaglandin $H_2$ ($PGH_2$); PGE synthase acts on $PGH_2$ to produce $PGE_2$ and $PGF_{2}\alpha$ (Hokin and Hokin, 1955).

Prostaglandin $F_{2}\alpha$ synthesized in the uterus targets its receptors on LLC and stimulates the production of luteal $PGF_{2}\alpha$. This action by $PGF_{2}\alpha$ was shown to promote luteal regression and cell death resulting in an increase of intracellular calcium (Wiltbank et al., 1989a) activation of PKC, and a reduction of progesterone secretion from LLC. The PKA signaling pathway may be the predominant pathway for small luteal cells, but not the only $2^{nd}$ messenger system LH uses to control gene activation (Herrlich et al., 1996). Both PKA and PKC pathways have been found to use similar down-stream second messengers to initiate various genomic responses within the cell (Davis et al., 1987; Wiltbank et al., 1990; Niswender, 2002). Crosstalk has been suggested to occur between these two signaling pathways, after constitutive binding of LH occurred in both small and large luteal cells activating PLC, and resulted in an increase of $IP_3$ and $Ca^{2+}$ in the cow (Davis et al., 1987). The $PGF_{2}\alpha$

receptor (FP) mRNA has been shown to be extremely low in theca or granulosa cells, but highly expressed in the CL, particularly in the LLC. Receptors for $PGF_2\alpha$ were characterized and reviewed by Anderson et al. (2001) throughout the estrous cycle in *murine*, and *bovine* species and throughout the menstrual cycle in humans, revealing an increase in PG receptors in LLCs and a decrease in theca or granulosa cells. More recent evidence has demonstrated the existence of a $PGF_2\alpha$ transporter necessary to move the $PGF_2\alpha$ from the uterine vein to the ovarian artery. Because of PG's charged anions, they diffuse poorly across the plasma membrane, and must be transported for more efficient or rapid cell signaling; this carrier mediated transport of $PGF_2\alpha$ was found to be essential to facilitate the luteolytic action of $PGF_2\alpha$ (Schuster, 1998; Banu et al., 2003). Prostaglandin transporters (PGT) were detected to be highly modulated during different phases of the life span of the CL in the *bovine* (Arosh et al., 2004) and PGs exert their effects through their GPCR designated prostaglandin $E_2$ (EP) and FP receptors.

Metabolism of $PGE_2$ and $PGF_2\alpha$, is well known to be integrated playing key roles in the function of the CL (Arosh et al., 2004; 2016). The carrier transport system protects these PGs from being catabolized in the general circulation (Banu et al., 2003; Banu et al., 2008) and is necessary for exerting their biological effects when target cell receptors are activated in the PM of LLCs. However, how the prostaglandin transporter moves through the PM is poorly understood.

Interferon Tau (IFNT) produced by the ruminant conceptus was found to suppress production of $PGF_2\alpha$ by down regulating estrogen (ESR1) and oxytocin receptors (OXTR) located in the endometrium of sheep and cows (Spencer et al., 1996) thus preventing luteolysis and maintaining pregnancy.

Prostaglandin $E_2$ may also exert secondary luteal protective effects throughout the duration of pregnancy. Both prostaglandin $E_2$ and $PGF_2\alpha$ have been shown to work in unison to regulate the growth and regression of the CL by activating different protein kinase pathways to either stimulate or diminish the production of progesterone (Arosh et al., 2016). However, a better understanding of how local factors activate the "molecular switch" that ultimately controls luteolysis and therefore the estrous cycle in mammals, is needed. Progesterone was also shown to activate $Ca^{2+}$ release through $IP_3$ receptors (Hwang et al., 2009). It is possible that $IP_3$ binding proteins may also be involved in regulating luteolysis and the estrous cycle. Although much research has been accomplished, several questions remain unanswered.

### *Activation of Phosphatidylinositol Hydrolysis Pathway and the Secretion of OT in Ovine and Bovine CL*

As previously mentioned, OT is synthesized in the hypothalamus and stored in the posterior lobe of the pituitary, however, the CL has also been found to synthesize and secrete OT in the ewe (Wathes and Swann, 1982) and cow (Wathes et al., 1983). It is well known that OT is involved in promoting luteolysis and is also known to

regulate smooth muscle contractions in the uterus, and the ejection of milk from the mammary gland. Genes transcribed for OT during metestrus (day 1-4) were found to result in peak plasma OT concentrations between day 10-12 of the estrous cycle (Schams et al., 1982), suggesting that luteal OT must be replenished throughout the estrous cycle.

Prostaglandin $F_2\alpha$ was found to stimulate the release of luteal OT *in vivo* in the ewe (Flint and Sheldrick, 1982) and *in vitro* in the cow CL (Abdelgadir et al., 1987). The secretion of OT was also shown to be stimulated by arachidonic acid in a dose-dependent manner when luteal tissue was incubated *in vitro* (Hirst, 1988). Schramm et al. (1983) studied the effects of $PGF_2\alpha$ on LLC specifically, while purified preparations of large and small luteal cells *in vitro* confirmed that only LLC produce OT (Rodgers, 1983).

The secretion of OT in *ovine* (Flint and Sheldrick, 1982) and *bovine* CL was discovered to coincide with the pulsatile release of uterine $PGF_2\alpha$ which occurs as a consequence of oxytocin activating the phosphatidylinositol hydrolysis pathway (See Fig 1). Five pulses of uterine $PGF_2\alpha$ in a 25 hr period is necessary to initiate luteolysis, while low amplitude pulses stimulate the release of OT secretory granules from the LLC. This release of OT from the ovary acts on the uterus to perpetuate a pulsatile release of $PGF_2\alpha$, allowing luteolysis to occur. McCracken (1984) proposed that OT from the posterior pituitary stimulates the release of $PGF_2\alpha$ from the endometrium. This interaction between hypophysial OT and uterine $PGF_2\alpha$ was

identified in both sheep and cows (Silvia et al., 1991) and is known as a "double" positive feedback loop. The uterine $PGF_2\alpha$ goes on to activate the secretion of luteal OT which in turn stimulates release of more uterine $PGF_2\alpha$ and consequently luteolysis (McCracken et al., 1999), reviewed by Stormshak (2003).

It was determined that neither the presence of LH nor $PGE_2$ affected OT production regardless of the dose or the stage of the estrous cycle (Harrison et al., 1987). In addition, secretion of progesterone was shown to be stimulated in the presence of *ovine* LH, but not OT, presenting evidence that OT and progesterone are stimulated independently of each other.

**Evidence for Luteal Progesterone Receptors: Genomic and Non-Genomic**

Irving Rothchild (1981) hypothesized that the progesterone synthesized in the CL may act locally by targeting receptors for progesterone in the cell that produced it, and thus controlling luteal cell function. The proof for intra-luteal regulation by progesterone has not yet been convincingly established; however, in the last few decades several studies have provided evidence supporting autocrine action. As example, experiments by Smith et al. (1994) and Bolden-Tiller et al. (2002) detected PR mRNA in both *ovine* and *bovine* CL and the PR was also detected in the CL of the human (Maybin and Duncan, 2004), but not in the rat (Cai and Stocco, 2005).

The CL is a complex endocrine gland that utilizes both intra- and inter-molecular signaling to regulate the estrous cycle. Currently, there are three known isoforms of a membrane or non-genomic progesterone receptor (mPR); alpha

(mPRα), beta (mPRβ), and gamma (mPRγ) (Rae et al., 1998; Zhu et al., 2003; Kowalik et al., 2018). Increasing data suggests that progesterone may function utilizing both a genomic and non-genomic receptor in the luteal cell (Ashley et al., 2009), contributing to the complex molecular interactions regulated by progesterone locally.

The first antiprogestin, RU-486 or mifepristone, was discovered in 1980 at the Roussel-Uclaf laboratory in France (Donaldson et al., 1993), and would later become the active ingredient in the "morning after pill" because of its ability to block the receptors for progesterone in the uterus. RU-486 has an affinity for both glucocorticoid and progesterone receptors, however, RU-486 was shown to have a stronger affinity for progesterone receptors (Mahajan and London, 1997).

Telleria and Deis (1994) injected RU-486 into the ovarian bursa in the rat and demonstrated an inhibitory and stimulatory effect on the production of progesterone during pro-estrus and on various days of pregnancy. These data convinced Telleria and Deis that progesterone could stimulate, but also inhibit its own production.

Rothchild (1996) first proposed that progesterone was a luteotropin and suggested that blocking PRs with RU-486 resulted in mediating luteal progesterone synthesis by varying forms of its progesterone receptor, PR-A and PR-B. Currently, specific membrane progesterone receptors (mPR-α, mPR-β, mPR-γ) have been found in *bovine* luteal cells and may be involved in regulating the life span of the corpus

luteum during the estrous cycle (Kowalik et al., 2018). Kowalik et al. (2018) examined the expression of membrane PRα, β and γ in the *bovine* corpus luteum during early, mid and late stages of the estrous cycle as well as the first trimester of gestation. Only mPRα protein changed during the estrous cycle being greatest on days 2 to 5 and lowest on days 17 to 20 of the cycle. No differences in mPRα were detected during pregnancy and there were no significant differences in the expression of mPRβ and mPRγ during the estrous cycle or during pregnancy. Although immunostaining for each isoform of the mPR in the CL was detected throughout the estrous cycle, strong positive staining was most prominent in the SLC. Whether or not the nuclear receptor is translocated to the plasma membrane when triggered by $Ca^{2+}$ or some other signal within the cell, is yet to be determined.

*Concept of Intraluteal Regulation by Progesterone*

It has been accepted that the pituitary, placenta, and intraovarian hormones control $PGF_2\alpha$ and the growth and regression of the CL (Schramm, 1983; Olofsson, 1994). Ovarian progesterone synthesis is under the control of pituitary and placental hormones, while gonadal steroids modulate the action of gonadotropins. However, less information is available for an autocrine or paracrine action by progesterone, reviewed by Telleria and Deis (1994) and Rothchild (1996).

In pregnant rats, spontaneous luteolysis is dependent on intraluteal regulation by both progesterone and $PGF_2\alpha$, described by Stocco and Deis (1998). Progesterone has also been shown to have an anti-luteolytic effect on *bovine* luteal cells, by

suppressing the onset of apoptosis, a significant component of luteal regression (Rueda et al., 2000). *Bovine* luteal cells cultured in the presence of PR antagonists RU-486 and onapristone caused an approximately 40% increase in apoptosis in the corpus luteum. These data suggest that progesterone suppresses onset of apoptosis in the corpus luteum via a PR-dependent mechanism.

Molecules regulating luteolysis most likely work in concert with progesterone synthesized by the CL to control luteolysis. To date, regulatory effector molecules such as $Ca^{2+}$ and $IP_3$, have been found to have both an internal and external origin to trigger signaling within the cell. External signals originating from the brain, or other distant tissues are transferred across the cell membrane into the interior of the cell, where they are transduced by sequential reactions leading up to transcription or translation of specific proteins. The molecular signaling pathways that progesterone uses to regulate luteolysis is still unknown (Hirst et al., 1986; Rothchild, 1996).

**Phosphoinositides**

Phosphoinositides, or cellular phospholipids, were discovered in the 1950's and found to activate signaling molecules in the plasma membrane (Berridge and Irvine, 1984). Phosphorylation or dephosphorylation of these phospholipids, either by protein kinases or protein phosphatases, respectively, have been shown to control the life cycle of the cell almost entirely (Balla, 2013), playing an important role in regulating aspects of metabolism, cell growth and differentiation. Of the various protein kinases, the Ser/Thr and Tyr specific enzymes are best characterized,

revealing a switch function involved in regulating cell membrane signaling pathways (Krauss, 2008). Among the phospholipids, phosphatidylinositol 4,5-bisphosphate ($PIP_2$) has been the most studied, and even though a minor portion of the population in the plasma membrane, $PIP_2$ was found to control major aspects of cell signaling. More recently, phosphoinositides have been described to also regulate the functional characteristics of several ion channels and transporters (Hille et al., 2015). After decades of research in this field, it has been determined that possible crosstalk between $PIP_2$ and ion channels may exist and may be intricately involved in the signaling pathways of many tissues. Much more research is needed in this area of study (Hille et al., 2015).

Phosphoinositide-specific phospholipase C (PLC) enzymes were discovered in the plasma membrane with a total of 3 classes based on their structure, β, γ, and δ (some showing 6 subtypes or isoforms) (Kadamur and Ross, 2013). Each one of these PLC enzymes have catalytic sites found in the X and Y domains cleaving polar head groups from inositol phospholipids (Rebecchi and Pentyala, 2000) and have been an area of interest for the past couple decades. Crystallographic studies of PLC-δ1 have been performed to gain new insights of the multi-domain arrangement and catalytic mechanisms of the PLC isoforms that work together, contributing to complex crosstalk resulting in varied cellular responses. Phospholipase C-δ1 is important for anchoring binding proteins to the plasma membrane (by use of a PH domain) in response to extracellular stimuli, allowing activation by GPCR's.

Phosphatidylinositol 4,5-bisphosphate was found to produce another 2nd messenger molecule, phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$) involved in playing key roles in regulating cell survival. PI3-Kinase (PI3-K) can be activated by βγ-subunits of G proteins or receptor tyrosine kinases (RTK) and upon activation result in the formation of $PIP_3$ that goes on to bind pleckstrin homology (PH) domains of various signaling proteins promoting the association with the membrane and activating cell growth or inhibiting apoptosis (Krauss, 2003).

Originally, PLC enzymes and their subtypes were thought to function as agonists, stimulating phosphoinositide metabolism and $Ca^{2+}$ signaling pathways, but it is now thought they may play an additional indirect role in regulating the estrous cycle as well (Franke, 2008). The coupling of hormones to their receptors, signal transduction pathways activate effector proteins (such as adenylate cyclase and PLC) to amplify the signal (Krauss, 2003). These pathways are regulated by the coupling of hormones, to activate or inactivate the conformational changes in the docking sites and subcellular localization of proteins. In the past couple decades, an inactive protein for PLC was discovered and may be indirectly involved in regulating the estrous cycle, by preventing the stimulation of PLC and ultimately the production of the eicosanoid that activates luteolysis.

*Phospholipase C Related Inactive Protein (PRIP)*

It is well known that the enzyme Phospholipase C (PLC) is responsible for the cleavage of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) associated with the plasma

membrane, to produce second messengers, $IP_3$ and DAG. These second messengers increase intracellular $Ca^{2+}$ and facilitate activation of protein kinases. More recently, a binding protein for $IP_3$ was identified by investigating $IP_3$ analogs and their binding capabilities, which led to the discovery of a new PLC-like protein, the novel Phospholipase C Related Inactive Protein (PRIP) (Hirata et al., 1989), defined as p130 (Kanematsu et al., 1992).

The discovery of the $IP_3$ binding protein, PRIP, was made possible by use of affinity matrices, established by coupling $IP_3$ analogs to a supporting matrix comprised of Sepharose 4B (Hirata et al., 1990). In order to investigate the binding capacity of $IP_3$, the binding activity of three known $IP_3$ interactive macromolecules were absorbed onto affinity columns; two enzymes associated with $IP_3$ metabolism, $IP_3$ 5-phosphatase and $IP_3$ 3-kinase and the $IP_3$ receptor, were used. Rat brain samples were then applied to prepared columns and fractions were further analyzed by gel filtration, showing $IP_3$ binding to unknown molecules observed in two separate elution peaks. Eluates were analyzed by SDS-PAGE, and molecular weights were found to be 85 and 130 kDa in size. These scientists went on to partially sequence the amino acids for the two molecules, revealing that the 85 kDa molecule was in fact the delta isozyme of PLC (PLC-δ1) (Kanematsu et al., 1992). However, no known proteins to date, were identified for the amino acid sequences contained in the 130 kDa molecule. To ensure that interacting proteins or proteolytic products were not being detected, both rat and *bovine* brain fractions were analyzed through $IP_3$ affinity

columns (2 M NaCl). Recognized metabolic products in the rat brain, such as $IP_3$ 5-phosphatase, were known to be either 60 and 160 kDa or 32 and 69 kDa in size, and in the *bovine*, 51 and 115 kDa, which were not detected in the eluate. Kanematsu et al. (1992) concluded that a novel $IP_3$ binding protein existed in the rat brain, p130, and would also be described as PLC-L or PRIP.

The pleckstrin homology domain (PH domain) was first identified in the platelet protein pleckstrin, by Harlan et al. (1994), who established that the N-terminal portion of the protein consisting of the PH domain, bound to $PIP_2$ inspiring the in-depth study of the phosphoinositides as a potential target of the binding domain. However, several other regulatory molecules have since been discovered. Not all PH domains bind lipids, but likely all PH domains bind proteins (Lemmon, 2004) and have even been shown to interact with small GTP binding proteins, as reviewed by Falkenburger et al (2010).

In an attempt to determine the region where PRIP binds $IP_3$, transfected COS-1 cells with genes encoding PRIP, or several truncated mutants of the gene, were generated. Deleting N-terminal residues (containing the PH domain) from PRIP proteins, prevented the molecule from binding $IP_3$ molecules, confirming the binding site for $IP_3$ in the PH domain (Takeuchi et al., 1996; 2000). This new protein family was determined to play a regulatory role in the cell, based on its ability to bind to $IP_3$ while lacking PLC activity on $PIP_2$ and inositol phosphates.

Phospholipase C Related Inactive Protein's domain organization was discovered to be homologous to that of the PLC-δ1 enzyme, with 38% sequence identity. Three known domains of PLC-δ1 exist, the PH domain and the catalytic X and Y domains. These domains are 35.2%, 48.2%, and 45.8% homologies to PRIP, respectively (Kanematsu et al., 1996). Because of their similar domain organization, these binding proteins are likely to fold similarly.

However, PRIP differs from PLC-δ1 not only in molecular weight, but also with variations to amino ($-NH_2$) and carboxyl (-COOH) ends. The $-NH_2$ terminal end of PLC-δ1 contains conserved regions of histidine (His). Contrary to PLC-δ1 however, PRIP has been shown to contain mutations in the amino acids critical for PLC activity. These mutations located in their catalytic domain (X or Y) region gives rise to PRIP's intrinsic inactive properties.

Otsuki et al. (1999) was first to isolate cDNA encoding a second PRIP protein, changing the nomenclature from PRIP/PLC-L/p130 to PLCL1 or PLCL2. Phospholipase Inactive Protein 2 is also homologous to the binding domain organization of both PLCL1 and PLC-δ1. In various tissues in the body, it was determined that PLCL1 and PLCL2 are from different genes and not only a slicing isoform of PLCL1. The PH domain of this new protein was also shown to bind strongly to $PIP_2$ and $IP_3$. PLCL2 was shown to predominantly localize to the perinuclear areas in both myoblast and myotube C2C12 cells, suggesting that PLCL2 plays an important role in the regulation of $IP_3$ around the ER where the $IP_3$ receptor

exists. When PRIP1 or 2 detach from the PM in response to an increase of $IP_3$, the action of PLC and its downstream effects are enhanced. Otsuki determined the Kd value for PLCL2 binding to $PIP_2$ and $IP_3$, to be 3.7 μM and 2.5 μM, respectively. These scientists concluded that PLCL2 may be responsible for regulating phosphoinositide signaling, because like PLC-δ1, it is able to interfere with both inositol phospholipids and inositol phosphates. These two subfamilies for PRIP, have also been confirmed by Uji et al. (2002), utilizing northern blots and RT-PCR techniques, analyzing PRIP (1 and 2) mRNA in rat tissues. PRIP-2 was detected in more tissues than PRIP-1 and was identified as being more ubiquitous.

Phospholipase C Inactive Proteins have been shown to inactivate PKC, by reducing the release of intracellular $Ca^{2+}$ shown by using KO mice for PLC-L2 in β-lymphocytes. The PLC-L2 gene deficient mice grew to adulthood and appeared normal, but when B cells were examined, they showed an increase in intracellular $Ca^{2+}$, concluding that PLC-L2 is a novel regulator of B cell receptor signaling and immune response (Takenaka et al., 2003). Hwang et al. (2009) studied the effects of progesterone on $IP_3$ receptors in hippocampalneurons, and observed effects on the intracellular $Ca^{2+}$ signaling and nuclear translocation of phosphorylated Akt (PKB), showing that progesterone potentiated $IP_3$ receptor mediated intracellular $Ca^{2+}$ responses. Around the same time, Gao et al. (2009) analyzed the pleckstrin homology (PH) domain, in both PLCL1 and PLCL2, and studied their binding capacity to the $PIP_2$ molecule, utilizing cloned double KO mice for PLC-L1 and 2.

The PH domain of PLCL2 was shown to bind strongly to that of $PIP_2$ and $IP_3$ (Tsutsumi et al., 2011).

Lastly, Muter et al. (2016) has shown that chelating $IP_3$ with PLCL1, limits the release of calcium from the expanding ER, preventing calcium from accumulating in the mitochondrial matrix and preventing apoptosis in endometrial stromal cells. PLCL1 was also shown to regulate the activity of the major signaling transduction pathway, through silencing of PLC signaling in response to progesterone. Withdrawal from the binding protein triggers tissue breakdown and results in menstruation and the failure to maintain pregnancy. Knock down experiments in undifferentiated stromal cells determined that PLCL1 maintains basal P3K/PKB activity, preventing nuclear translocation of the transcription factor fork head box protein 01 and induction of the apoptotic activator BIM. In conclusion, Muter et al. suggests that PRIP-1 is capable of uncoupling PLC activation, reducing $PIP_2$ signaling, and preventing the influx of intracellular $Ca^{2+}$ by $IP_3$. How progesterone might be targeting the $PIP_2$ pathway in the corpus luteum is not yet elucidated. How prostaglandin and progesterone interact with phosphoinositide hydrolysis to mediate the growth and regression of the CL throughout the cycle is the focus of the present research.

# POSSIBLE AUTOCRINE ACTION BY PROGESTERONE *IN VIVO* AND *IN VITRO* IN THE *OVINE* CORPUS LUTEUM

## Introduction

Rothchild (1981) advanced a hypothesis that progesterone, presumably by autocrine/paracrine action, regulates its own secretion and other functions of the corpus luteum. Besides the production of progesterone, the corpus luteum of the cow (Shemesh and Hansel, 1975) and ewe (Rexroad and Guthrie, 1979) synthesizes prostaglandin $F_2\alpha$ ($PGF_2\alpha$). Since $PGF_2\alpha$ has been established to be the major luteolysin in mammalian species the synthesis of this eicosanoid by the corpus luteum could be detrimental to its own life span if not somehow held in check. Rothchild (1981) proposed that progesterone exerts a suppressive effect on luteal prostaglandin production being strongest as the corpus luteum develops and weakening with advancing age. In this regard, Pate (1988) demonstrated using 24 h cultures of *bovine* luteal cells that progesterone caused a significant dose-dependent decrease in the synthesis of $PGF_2\alpha$ and 6-keto-$PGF1\alpha$ in cells derived from corpora lutea on days 6 and 10 but not 4 and 18 of the estrous cycle.

Prostaglandin $F_2\alpha$ has been shown to stimulate phospholipase C- catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate resulting in the generation of second messenger's diacylglycerol (DAG) and inositol trisphosphate ($IP_3$) in mature *bovine* luteal cells (Davis et al.1987; 1988). This biochemical process underlies the reported ability of $PGF_2\alpha$ to stimulate oxytocin secretion by the mature *bovine* (Abdelgadir et al., 1987) and *ovine* (Flint and Sheldrick, 1982) corpus luteum. While

it is important to note this action of $PGF_2\alpha$ on the corpus luteum of the ruminant, it is likewise important to note that $PGF_2\alpha$ is itself a product of phosphoinositide hydrolysis arising from the generated arachidonic acid within the CL. Experimental evidence acquired using *ovine* large luteal cells suggest that $PGF_2\alpha$ can act in an autocrine manner to stimulate its own production (Tsai and Wiltbank, 1997; Wiltbank et al., 2012). Similarly, Shirasuna et al. (2004) showed increased intraluteal $PGF_2\alpha$ production during spontaneous luteolysis in the cow.

If an autocrine role for progesterone exists by which it could regulate intraluteal $PGF_2\alpha$ production as suggested by Rothchild (1981) its effect would likely require binding to a receptor. Several progesterone receptors (PR) have been found to exist in the ruminant corpus luteum. Such as the classical nuclear PR (Rueda et al., 2000; Sakumoto et al., 2010) as well as the PGRMC1 form of the receptor, too (Luciano et al., 2011). Rae et al. (1998) detected a membrane PR using a digitonin enriched preparation of *bovine* luteal plasma membranes. These investigators showed that binding of progesterone was specific and not attributed to intercalation of the steroid into the membrane lipid bilayer. Luteal binding sites were specific for progesterone and not steroids with similar hydrophobicity. Ashley et al. (2009) have described an *ovine* luteal membrane PR$\alpha$ associated with the endoplasmic reticulum that specifically binds progestins. During the estrous cycle the expression of mRNA for the luteal progesterone receptor was greatest on day 10 compared to days 4 and 15. More recently, Kowalik et al. (2018) reported the presence of mPR$\alpha$, mPR$\beta$, and

mPRγ in the *bovine* corpus luteum during various stages of the estrous cycle. Only protein expression for mPRα changed during the estrous cycle being greatest early in the cycle and declining to lowest levels on days 17-20. The receptors were predominately located on SLC cells but were also expressed on the LLC.

In studies conducted to determine the action of progesterone on the CL, attempts have been made to block its action by RU-486. Ashley et al. (2009) and Davis et al. (2010) have concluded that RU-486 cannot block the binding of progesterone in luteal cells. In contrast, RU-486 has been effective *in vivo* in some cases. Morgan et al. (1993) showed that administration of RU-486 to ewes on day 5, 6, 7 and 8 of the estrous cycle, resulted in an extension of the cycle. Whether the effect of RU-486 was directly on the ovary or by an indirect mechanism was not evident. Rueda et al. (2000) also showed that RU-486 blocked the ability of progesterone to inhibit apoptosis in *bovine* luteal cells. These differences in response to RU-486 may have occurred due to a variation in concentration of antagonist used, tissue types, or other experimental conditions.

The present experiments were conducted to test the hypothesis, does progesterone inhibit phosphoinositide hydrolysis in the mature *ovine* corpus luteum? Therefore, changes in phosphoinositide hydrolysis in response to RU-486, the PR antagonist, and $PGF_2\alpha$, were assessed using both an *in vivo* and *in vitro* approach.

## Materials and Methods

*Experiment 1*

Ten mature Polypay ewes (3-5 yrs of age, weighing 65-85 kg) with normal estrous cycles (17 ± 1 days) were synchronized with CIDRs (Controlled Internal Drug Release) (Eazi-breed 0.3 g progesterone; Pfizer, New York, NY) inserted on day 1 and treated with 125 µg Estrumate (cloprostenol; Schering-Plough, Union, NJ) im on day 6 to regress corpora lutea. CIDRs were removed on day 7 with estrus detected by use of a vasectomized ram 24-48 hr later. At detected estrus (day 0 of the cycle) ewes were assigned randomly to a control and treatment group consisting of 5 animals each. Ewes were monitored for behavioral estrus using a vasectomized ram throughout the remainder of the cycle. All experimental procedures and protocols were reviewed and performed in accordance with the institutional Animal Care and Use Committee guidelines at Oregon State University.

On day 8 of the estrous cycle, a jugular blood sample (6 mL) was taken and surgery was performed. Ewes were anesthetized with an iv injection of Ketamine, 2 mL/100 lb (Fort Dodge, Overland Park, KS) and diazepam, 1 mL/100 lb (Hospira, Lake Foreset, IL) followed by maintenance of anesthesia via inhalation of a mixture of oxygen and isoflurane (Isothesia: Abbott Laboratories, Chicago, IL).

Treated ewes were given RU-486 (10 µg) dissolved in 1 mL saline with 0.2% ethanol injected into the ovarian artery supplying an ovary bearing the corpus luteum (CL). Likewise, control ewes received a 1 mL injection of vehicle of 0.9% NaCl

containing 0.2% of ethanol into the ovarian artery also supplying an ovary bearing a CL. Jugular blood samples were collected 10 min post ovarian artery injections from both treated and control ewes. Blood samples were stored on ice until later centrifuged at 3000 x g for 10 min at 4°C and the serum then stored at -20°C until assayed for progesterone. The CL was enucleated from the ovary and immediately placed into cold Krebs Ringer Bicarbonate (KRB) gassed with 5% $CO_2$ -95% $O_2$ and transported to the laboratory.

The CL obtained from ewes were kept on ice and sliced into 50 mg $\pm$ 5 mg aliquots. Triplicate aliquots of tissue from each ewe placed into flasks containing 1 mL of cold oxygenated KRB to which had been added 10 µCi $^3$H-myo-inositol (23.8 ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) were incubated for 90 min at 37°C. Krebs Ringer Bicarbonate medium was then removed and 1 mL oxygenated KRB (37°C) was added to each sample. Twenty µL of 2.55 M lithium chloride (LiCl) was added to each sample, gassed and incubated for 10 min at 37°C at which time 20 uL of 10 nM $PGF_2\alpha$ (Estrumate) or saline were added to flasks containing tissue from control and RU-486 *in vivo* treated ewes. This experimental design resulted in a factorial arrangement of treatment groups consisting of: 1) Control, 2) $PGF_2\alpha$, 3) RU-486 and 4) RU-486 + $PGF_2\alpha$. Samples were gassed and incubated at 37°C for 15 min. Incubation was terminated by placing the samples in an ice bath. Krebs Ringer Bicarbonate was removed from each vial and 1 mL ice cold 15% TCA was added to the remaining tissue on ice for 30 min. Trichloroactetic

acid was collected and placed into 15 x 85 mm glass tubes, the tissue in the vial was rinsed with 200 µL dd$H_20$ and added to the tube containing the TCA. Water-saturated diethyl ether (5 mL) was added to TCA media, tubes were capped and shaken vigorously for 5 s, and ether was then removed and discarded for a total of 5 diethyl ether extractions. Finally, residual ether was removed with $N_2$ gas for 5-10 min in a 37°C water bath. Trichloroacetic acid was neutralized with 25 µL 0.5 M Tris-HCl and pH checked with litmus paper. Samples were stored at -20°C until analyzed for activity of tritium labeled inositol phosphates.

*Experiment 2*

The *in vitro* responses of luteal tissue in Experiment 1 were a consequence of *in vivo* exposure of tissue to RU-486 and *in vitro* exposure to $PGF_2\alpha$. Therefore, Experiment 2 was conducted to confirm the responses of luteal tissue to RU-486 and $PGF_2\alpha$ using an entirely *in vitro* approach. For this experiment, 5 Polypay ewes were estrous synchronized as described for Experiment 1. On day 8 of the estrous cycle (estrus=day 0 of the cycle) ewes were anesthetized as described above and corpora lutea collected by midventral laparotomy. Corpora lutea were weighed and sliced as described in Experiment 1. Triplicate 50 mg aliquots of tissue from each ewe were placed into flasks and subjected to the following sequence of incubation conditions as defined for Experiment 1: 1) 90 min incubation in 1 mL oxygenated KRB containing 10 µCi $^3$H-myo-inositol, 2) removal of medium and addition of 1 mL oxygenated KRB containing 2.55 M LiCl with incubation for 10 min, 3) followed by addition of

$PGF_{2}\alpha$ (1 μM), RU-486 (2 μM), RU-486 + $PGF_{2}\alpha$ or vehicle and incubation for another 15 min. $PGF_{2}\alpha$ and RU-486 were dissolved in saline + 0.2% ethanol and 20 μL of each or the vehicle were added to the KRB. All incubations were performed at 37°C. Samples were processed as described for Experiment 1 to recover and quantify $^{3}$H-myo-inositol phosphates.

*Inositol Phosphate Assay*

Inositol phosphates were separated out of TCA solutions (stored at – 20°C) by use of Poly-Prep columns (Bio-Rad Laboratories Inc., Hercules, CA) containing (1 mL) analytical grade type 1-X8 anion-exchange resin (Formate Form, 200-400 mesh; Bio-Rad Laboratories Inc.) and washed with 2 mL deionized water with rinses being applied to the column. Columns were eluted with an additional 8 mL deionized water and discarded. Columns were then washed with 10 mL $NH_4$ formate (50 mM) and eluted into waste chamber. Samples (1 mL) were added to columns and 6 mL $NH_4$ formate (1.2 M)/ formic acid (0.1 M) solution were added and samples collected into scintillation vials. Each sample collected was measured for incorporation of $^{3}$H-myo-inositol by methods originally reported by Mirando et al. (1990). Samples were in vials containing 6 mL liquid scintillation cocktail (Ecolume, MP Biomedicals, LLC, Solon, OH) and labeled phospholipids were quantified by liquid scintillation spectrometry in a Beckman LS 6500 scintillation counter (Fullerton, CA).

*Progesterone Extraction and RIA*

Progesterone was measured by radioimmunoassay and steroids extracted with 2 mL benzene: hexane (2:1), from serum samples (100 µL) collected in experiment 1 and media samples (150 µL:100x) from experiment 2. Standard curve values ranging between 5 pg-800 pg were used and samples were run in triplicate with quality controls dispersed throughout the assay. The extraction efficiency was calculated for the first few samples and was within 95%. Serum and media samples were vortexed for 2.5 min with benzene: hexane (2:1) and stored at -20°C overnight. Twenty-four hr later the benzene: hexane portion of the sample was poured into a clean tube and dried down under air. Progesterone antiserum (#337 Anti-$P_4$-11-BSA Serum, Colorado Springs, CO) was diluted to 1:2,400 and 100 µL were added to samples and incubated for 30 min. Competitor (100 µL) (21,000 cpm $^3$H-$P_4$; 96.6 Ci/mmol, Perkin Elmer Life and Analytical Sciences) was added and tubes were vortexed for 20 sec and incubated overnight at 4°C. On the following day, 1 mL dextran-coated charcoal was added, and tubes were centrifuged at 4°C at 3,000 x g for 10 min, poured into scintillation vials with 6 mL Ecolume and counted on the Beckman LS 6500. Intraassay coefficients of variation (CV) were calculated from quality control standards interspersed throughout the assay and resulted in the average CV values of 0.98% in Exp 1 and 3.4% in Exp 2.

*Statistical Analysis*

Data on incorporation of $^{3}$H-myo-inositol into phosphoinositide phosphates and incubation medium concentration of progesterone were analyzed using repeated measures analysis of variance. Data on serum concentration of progesterone present before and after treatment were analyzed statistically by analysis of covariance to adjust for number of corpora lutea present in each animal. When significant F values were obtained by analysis of variance, differences among means were tested for significance using a Duncan's New Multiple Range Test.

# RESULTS

## Experiment 1

This experiment was conducted to compare the effect of RU-486 and $PGF_{2}\alpha$ on phosphatidylinositol hydrolysis in the mature corpus luteum of the ewe on day 8 of the estrous cycle. Corpora lutea recovered from control and *in vivo* RU-486 treated ewes were subsequently exposed to 10 nM $PGF_{2}\alpha$ during *in vitro* incubation resulting in factorial arrangement of experimental groups. Injection of 10 μg RU-486 into an ovarian artery supplying an ovary bearing a corpus luteum activated phosphatidyl-inositol hydrolysis in the recovered luteal tissue during incubation. Incorporation of 10 μCi $^{3}$H-myo-inositol into phosphatidylinositol hydrolysis pathway phosphates increased in response to RU-486 alone, $PGF_{2}\alpha$ alone and a combination of the two hormones compared to that of controls (Fig 1). Mean increase in incorporation of myo-inositol was maximal in response to $PGF_{2}\alpha$ but did not differ significantly from

those for RU-486 or RU-486 + $PGF_2\alpha$. Similarity in positive responses to $PGF_2\alpha$ and RU-486 resulted in a RU-486 x $PGF_2\alpha$ interaction ($p<0.05$).

Administration of either 1 mL of RU-486 or vehicle into the ovarian ovary resulted in a significant reduction in serum progesterone concentration by 10 min after injection (Fig 2). Mean serum concentrations of progesterone prior to and 10 min after injection were as follows for control, 3.24 and 1.11 ng/mL, and RU-486 treated ewes, 3.38 and 2.09 ng/mL ($p<0.05$).

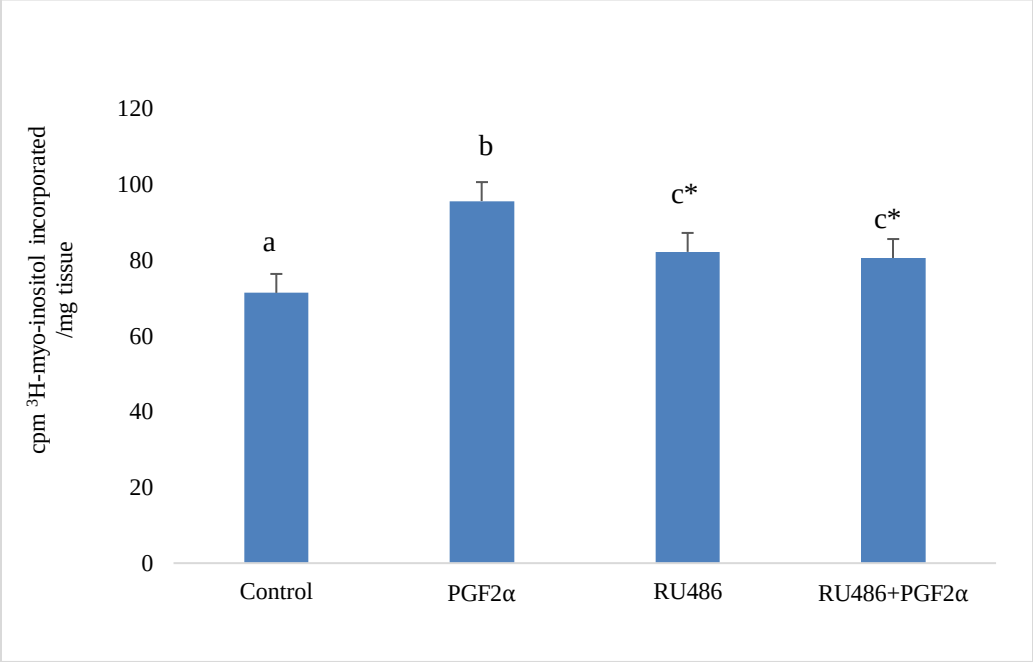


Fig 2. Effects of RU-486 and $PGF_2\alpha$ injected into the ovarian artery on *in vitro* incorporation of myo-inositol into luteal phosphoinositides. [a,b,c] Means (±SE) differ at $p<0.05$ and $p<0.07$*.

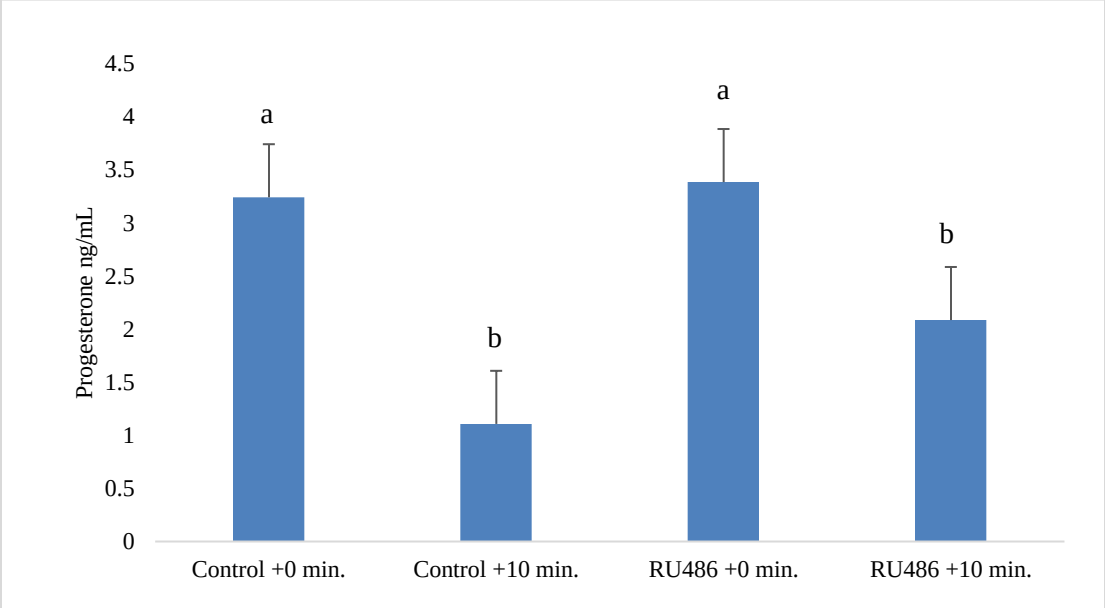


Fig 3. Serum progesterone levels obtained from blood sampling before and after an injection of saline (control) and RU-486 into the ovarian artery on day 8 of the estrous cycle. [a,b] Means (±SE) differ, $p<0.05$.

**Experiment 2**

Experiment 2 was conducted to confirm the results of Exp 1. Luteal tissue collected on day 8 of the estrous cycle was subjected to a 2x2 factorial arrangement of treatments with RU-486 (2 µM) and $PGF_2\alpha$ (1µM). Compared to control, both RU-486 and $PGF_2\alpha$ alone and in combination significantly increased incorporation of $^3H$ myo-inositol into phosphoinositide phosphates (RU-486 x $PGF_2\alpha$ interaction, $p<0.25$; Fig 3). As in Exp 1, $PGF_2\alpha$ stimulated the greatest incorporation into

phospholipids. Mean level of incorporation stimulated by RU-486 alone or in combination with $PGF_2\alpha$ did not differ but was markedly greater than that of control.

Progesterone levels detected in the incubation medium are presented in Fig 4. By comparison to controls, levels of progesterone increased in response to exposure of luteal tissue to RU-486 and were significantly increased by $PGF_2\alpha$ ($p<0.05$) in just 15 min.

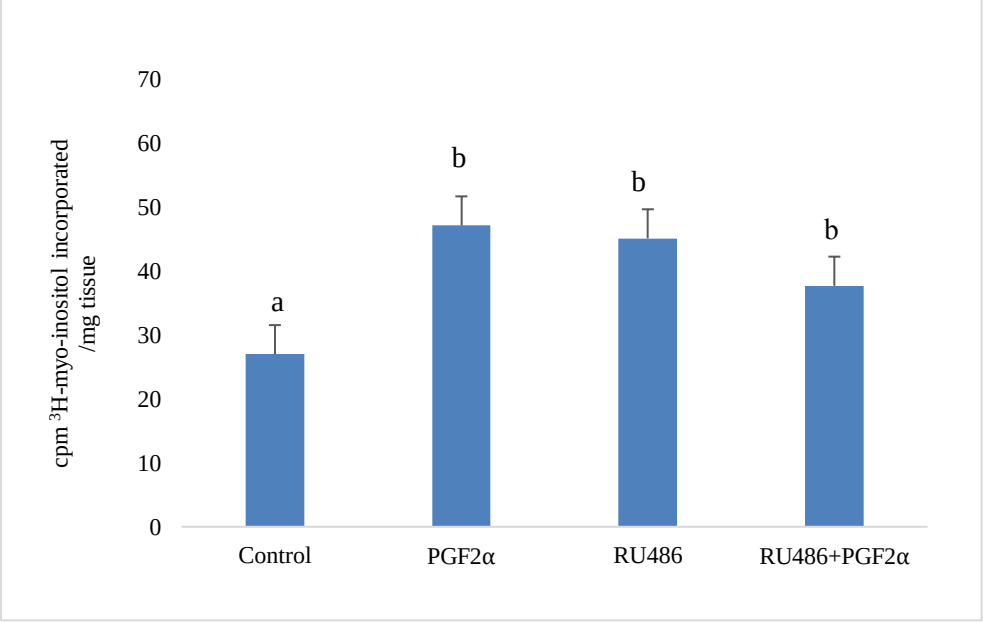


Fig 4. Effects of RU-486 and $PGF_2\alpha$ on *in vitro* incorporation of myo-inositol into luteal phosphoinositides (RU-486 x $PGF_2\alpha$ interaction, $p<0.025$). $^{a,b}$ Means ($\pm$SE) differ, $p<0.05$.

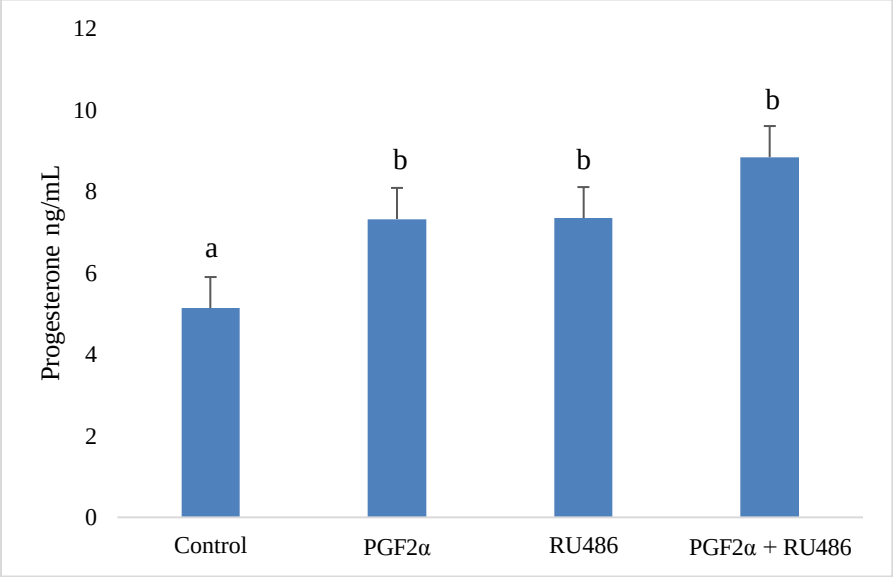


Fig 5. Progesterone concentration in medium after 15 min incubation of luteal tissue exposed to vehicle (control), $PGF_2\alpha$, RU-486 alone or in combination. [a,b] Means (±SE) differ, $p<0.05$.

# DISCUSSION

In the present studies, short term exposure of *ovine* luteal tissue on day 8 of the estrous cycle to RU-486 or $PGF_2\alpha$ activated phosphatidylinositol hydrolysis. Exposure of luteal tissue to RU-486, either by injection into the ovarian artery or during *in vitro* incubation stimulated phosphatidylinositol hydrolysis as evidenced by incorporation of $^3$H-myo-inositol into phosphatidylinositol phosphates. The observed responses are believed to be due to a rapid non-genomic effect of RU-486 and $PGF_2\alpha$. In the case of RU-486, an increase in inositol phosphates occurred in just 10 minutes exposure to the antagonist *in vivo* and 15 min *in vitro*.

It is presumed that the observed responses to RU-486 represents the ability of this synthetic steroid to block the suppressive action of progesterone on phosphatidylinositol hydrolysis. It also may be assumed that the rapid response to RU-486 is a consequence of its binding to membrane progesterone receptors (mPR). Indeed, a mPR has been found to exist in the *ovine* (Ashley et al., 2009) and in the *bovine* corpus luteum (Rae et al., 1998). However, these investigators could find no binding affinity of RU-486 to the mPR. On the other hand, Bottino et al. (2011) reported the presence of nuclear progesterone receptors (nPR) A and B tethered to the plasma membranes in murine mammary carcinomas. *In vitro* exposure of carcinoma cells to concentrations of medroxyprogesterone acetate (MPA) and RU-486 lower than $1 \times 10^{-11}$ M bound to the membrane associated nPR and activated the MAPK pathway. These investigators also found that tumors express the non-classical mPR

α, β, and γ but again these receptors did not bind MPA, RU-486 or the progesterone agonist R5020. In human T cells progesterone is an immunomodulator that suppresses activation of these cells during pregnancy. Chien et al. (2009) reported the existence of mPRα, β, and γ in T cells and found that RU-486 acted in a non-genomic manner to increase intracellular $Ca^{2+}$ and decrease pH. Thus, whether RU-486 can bind to mPRs to activate non-genomic responses is equivocal. The observed differential *in vitro* responses to RU-486 may be due to experimental conditions. Alternatively, RU-486 may be binding to a membrane associated nPR or receptor yet to be identified.

*In vitro* effect of prostaglandin Fα on luteal tissue phosphatidylinositol hydrolysis was examined for comparison purposes in experiments 1 and 2. It is well known that in sheep and cows the developing corpus luteum during days 1 to 6 of the estrous cycle is resistant to $PGF_2\alpha$ induced luteolysis (Louis et al., 1973; Acritopoulou and Haresign, 1980; Rubianes et al., 2003). However, during the mid to late luteal phase of the cycle, exposure of the corpus luteum to a sufficient amount of exogenous or endogenous $PGF_2\alpha$ results in luteolysis (Louis et al., 1974; Inskeep et al., 1975). The mature corpus luteum of the cow and ewe synthesizes $PGF_2\alpha$ as determined by the presence of quantifiable levels of this eicosinoid (Shemesh and Hansel, 1975; Rexroad and Guthrie, 1979). It has been reported that $PGF_2\alpha$ can by autocrine action stimulate its own synthesis by *bovine* luteal cells (Tsai and Wiltbank, 1997; Shirasuna et al., 2004). Such action by $PGF_2\alpha$ would require stimulation of

phosphatidylinositol hydrolysis in order to generate arachidonic acid essential for prostaglandin synthesis. Indeed, results of the present experiments are supportive of such action by $PGF_2\alpha$. In both experiments $PGF_2\alpha$ increased incorporation of $^3H$-myo-inositol into phosphoinositide phosphates. This scenario of the autocrine action of prostaglandin then begs the question; "What prevents the mid-cycle corpus luteum from self-destructing?"

Our data suggests that the activation of the pathway leading to phosphatidylinositol hydrolysis with consequent production of $PGF_2\alpha$ may be inhibited by progesterone at least during the mid-luteal phase of the cycle. Such an action by progesterone is consistent with the original hypothesis proposed by Rothchild (1981) that progesterone can act in an autocrine fashion. This is also consistent with his proposition that progesterone inhibition of $PGF_2\alpha$ is stronger during the mid versus late luteal phase of the estrous cycle. This action of progesterone is also supported by the research of Pate et al. (1988) who found that exogenous progesterone was shown to inhibit luteal $PGF_2\alpha$ synthesis when *bovine* CLs were cultured on d 6 and 10 but not on d 4 and 18 of the estrous cycle. Davis et al. (2010) have also reported a suppressive effect of progesterone on small luteal cells in the *ovine* corpus luteum. Treatment of these small luteal cells in culture with progesterone for 1 h suppressed an oxytocin induced increase in intracellular calcium. In these cells oxytocin may have stimulated the phosphatidylinositol pathway thus generating an inositol trisphosphate ($IP_3$) induced increase in intracellular $Ca^{2+}$.

The ability of progesterone to regulate phosphoinositide hydrolysis has been shown to occur in other reproductive organs as well. Incubation of *ovine* endometrial plasma membranes with progesterone for 1 h inhibited binding of oxytocin to its receptor; whereas this effect of progesterone was blocked by RU-486 (Dunlap and Stormshak, 2004). Endometrial plasma membranes were examined for presence of a progestin binding site using labeled progesterone and agonist R5020. Analysis of binding data by Scatchard plot revealed the presence of a high affinity binding site for progesterone and R5020 (Kd $1.2 \times 10^{-9}$ and $1.74 \times 10^{-10}$ M, respectively). Subsequently, Bishop and Stormshak (2006) found that *ovine* endometrial explants exposed to progesterone for as little time as 10 min interfered with oxytocin stimulation of $IP_2$ and $IP_3$ synthesis as well as synthesis of prostaglandins. Once progesterone is bound to its receptor it may be possible that progesterone is involved in the regulation of $PGF_2\alpha$ synthesis by utilizing the $IP_3$ binding protein, Phospholipase C- Related Inactive Protein (PRIP). This binding protein when activated inhibits the action of PLC thereby preventing the activation of the $PIP_2$ pathway and its second messengers. Muter et al. (2016) showed that progesterone induced PRIP proteins uncouple PLC activation from intracellular calcium release by attenuating $IP_3$ signaling. This may or may not be the mechanism by which progesterone acts in the CL.

Treatments *in vivo* and *in vitro* with RU-486 and $PGF_2\alpha$ have affected luteal production of progesterone. In experiment 1, serum concentration of progesterone in

ewes on day 8 of the estrous cycle prior to treatment were comparable to those reported by Karsch et al. (1980). Injection of 1 mL of saline alone or containing 10 μg of RU-486 caused a significant reduction in serum levels of progesterone in 10 min. Although the capillary system of the *ovine* CL only accounts for approximately 14% of the mass of the corpus luteum in the mid-cycle (Niswender et al., 1976), we speculate that the progesterone in the systemic circulation was reduced due to the 1 mL saline or RU-486 injected into the ovarian artery causing a washout effect in the corpus luteum.

In experiment 2, progesterone levels were increased by exposure of luteal tissue to RU-486 and significantly increased by $PGF_2\alpha$. This stimulatory effect of $PGF_2\alpha$ on progesterone production is in accord with the data published by Speroff and Ramwell (1970). It should be noted that the measured progesterone in the present experiment represents the final concentration of steroid after 15 min exposure to RU-486 and/or $PGF_2\alpha$. The quantity of progesterone present in the incubation medium prior to addition of treatments could not be determined but is assumed to have been less than the final mean control level of 5.14 ng/mg tissue. It is possible in the present experiment that progesterone levels at initial exposure to RU-486 (2 μM) ng) and $PGF_2\alpha$ (1 μM) were too low to block the ability of these agents to activate the phosphoinositide pathway.

In summary, the results of the present *in vivo* and *in vitro* experiments demonstrate that the progesterone antagonist RU-486 can activate phosphoinositide

hydrolysis in the *ovine* corpus luteum. The observed rapid responses to the progesterone antagonist RU-486 suggest that progesterone can act in an autocrine/paracrine nongenomic manner to inhibit phosphatidylinositol hydrolysis during the midluteal phase of the estrous cycle. By this action progesterone may prevent the corpus luteum from self-destructing through suppressing its production or action of the luteolysin, prostaglandin $F_{2}\alpha$.

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