AN ABSTRACT OF THE THESIS OF

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Title: **Cyclopropenoid Fatty Acid-Induced Suppression of Ovine Corpus Luteum Function**

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Dr. Fredrick Stormshak

Three experiments were conducted to examine the effect of sterculic acid (SA), a cyclopropenoid fatty acid, on ovine luteal function. In Exp. 1, 12 ewes were laparotomized on day 10 of the estrous cycle and ewes with corpora lutea (CL) in both ovaries were unilaterally ovariectomized. An extract of *Sterculia foetida* seeds (1.09 mg) containing 750 ug SA methyl ester (n = 6) or 1.09 mg oleic acid methyl ester (OA; n = 6) were injected into the artery supplying the ovary bearing the CL. Jugular blood was collected for progesterone (P_4) radioimmunoassay 12 h post- surgery and then daily until day 13 of the cycle or until ewes exhibited estrus, whichever came first. In Exp. 2, six ewes were laparotomized on day 10 of the cycle and CL were removed, weighed and sliced. Luteal slices were incubated (37° C in Ham's F-12 medium under 95% O_2-5% CO_2) with 106 ng/ml of either SA or OA methyl ester for 90 min. Medium was

then replaced with fresh medium to which was added vehicle, 40 ng ovine LH (oLH; final concentration 10.67 ng/ml), or 18.7 mg dbcAMP (10.64 mM final concentration) and the tissue was reincubated for 90 min. Tissue plus medium were assayed for P_4. In Exp. 3, 11 ewes were laparotomized on day 10 of the cycle and injected with SA (n = 6) or OA (n = 5) as described for Exp. 1. Thirty hours later ewes were relaparotomized and CL were collected for LH receptor assay using ^{125}I-hCG as the labeled ligand. *In vivo* treatment with SA reduced serum concentrations of P_4 (ng/ml) within 12 h after treatment (mean + SE; control, 2.3 ± 0.4 vs. treated, 0.3 ± 0.1) and thereafter the serum levels of P_4 remained suppressed ($P < 0.001$) Estrous cycles of treated ewes were shorter than those of controls (mean + SE; control, 16.9 ± 0.4 vs. treated, 14.8 ± 0.8 days; $P < 0.05$). Pretreatment of luteal slices with SA reduced *in vitro* P_4 synthesis when subsequently incubated with either oLH or dbcAMP (mean + SE, ng/mg tissue; OA-oLH, 41.5 ± 3.0 vs. SA-oLH, 27.9 ± 3.1; OA-dbcAMP, 38.0 ± 3.0 vs. SA-dbcAMP, 28.8 ± 2.8; $P < 0.05$). Injection of SA reduced unoccupied luteal plasma membrane LH receptor numbers compared with those in CL of OA controls (mean + SE; OA controls, 161.4 ± 10.8 vs. SA-treated, 82.8 ± 16.6 fmol/mg protein; $P < 0.05$). Binding affinities of the LH receptor were similar (mean $K_d = 2.45 \times 10^{-13}$ M and 2.23×10^{-13} M for OA control and SA-treated

ewes, respectively) indicating that integrity of the receptor was not affected by SA-treatment. Results of this research indicate that SA suppresses luteal steroidogenesis in mature CL both *in vitro* and *in vivo* with the *in vivo* results due, at least in part, to a reduction in unoccupied plasma LH receptors concentrations.

Cyclopropenoid Fatty Acid-Induced Suppression of Ovine Corpus Luteum Function

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Head of Department of Animal Science

Redacted for privacy

Dean of Graduate School

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CYCLOPROPENOID FATTY ACID-INDUCED SUPPRESSION OF OVINE CORPUS LUTEUM FUNCTION

REVIEW OF LITERATURE

Characteristics of the Mammalian Corpus Luteum in the Nonpregnant Animal

FORMATION, MORPHOLOGY & BIOCHEMISTRY OF THE CORPUS LUTEUM

The corpus luteum (CL) is an ephemeral endocrine organ formed from cells of the follicle following ovulation. Under the influence of luteinizing hormone (LH), granulosa and theca cells from the ovulated follicle undergo luteinization to form the developing CL in mammals.

The corpus luteum in a variety of domestic species including sheep (Mossman and Duke, 1973), cattle (Donaldson and Hansel, 1965) and pigs (Lemon and Loir, 1977) is composed of two distinct steroidogenic cell types. These cell types have been designated small and large (Fitz et al., 1982) and can be distinguished on the basis of morphological and biochemical criteria. Structural and histochemical observations support the concept that granulosa cells initially give rise to the developing large luteal cells, while the small cells originate from the thecal layer of the preovulatory

follicle (O'Shea et al., 1980). In the ewe, small luteal cells can be identified by their small size (12 - 22 um in diameter) and characteristic spindle or elongated shape (O'Shea et al., 1979; Fitz et al., 1982). These cells contain irregularly shaped nuclei, darkly staining cytoplasm, numerous mitochondria, large lipid droplets, and an abundance of smooth endoplasmic reticulum (O'Shea et al., 1979, 1980). In contrast, large luteal cells are spherical in shape, 22 - 40 um in diameter, and are characterized by a greatly folded plasma membrane associated with a prominent basal lamina, a sperical nucleus, numerous mitochondria, a small number of lipid droplets, isolated stacks of rough endoplasmic reticulum and an abundance of the smooth endoplasmic reticulum characteristic of steroidogenic cells (O'Shea et al., 1979, 1980). Large cells uniquely contain electron-dense, membrane bound secretory granules, which, based on immunocytochemical data appear to be the source of luteal oxytocin in the sheep (Rodgers et al., 1983a; Sawyer et al., 1986) and cow (Wathes et al., 1983). Relaxin has also been identified in similar granules in rats (Anderson and Sherwood, 1984), pigs (Belt et al., 1971; Kendall et al., 1978) and cows (Fields et al., 1980).

In addition to morphological differences, ovine small and large luteal cells differ in several biochemical parameters and have different steroidogenic capacities. Although both cell types secrete

progesterone, there is evidence to suggest differences in the systems regulating steroidogenesis in large and small cells. When large and small luteal cells were isolated and incubated separately, LH treatment caused a marked stimulation, approximately 20-fold, of progesterone secretion from small luteal cells but essentially did not affect progesterone secretion from large luteal cells (Fitz et al., 1982; Rodgers et al., 1983b). However, in the absence of LH, the basal secretion rate of progesterone from large luteal cells is approximately sevenfold greater than that of small cells (Fitz et al., 1982; Hoyer and Niswender, 1985). In small ovine luteal cells, progesterone production is stimulated by dibutyrl cyclic adenosine monophosphate (dbcAMP) or agents that activate adenylate cyclase such as cholera toxin and forskolin (Hoyer et al., 1984; Schwall et al., 1985; Niswender et al., 1985). These data suggest that progesterone production by small cells is regulated by the cAMP second messenger system. The mechanisms controlling steroidogenesis in large cells is still unclear. Confusion regarding large luteal cells arises in part by the observation that, although cholera toxin or forskolin dramatically increased intracellular levels of cAMP and secretion of cAMP into the media, dbcAMP did not affect progesterone production (Hoyer et al., 1984; Schwall et al., 1985; Niswender et al., 1985). Although large luteal cells contain an active adenylate cyclase

system (Hoyer and Niswender, 1985) and have similar numbers of LH receptors as small cells (Harrison et al., 1987), the adenylate cyclase system of this cell type does not appear to be involved in regulating the synthesis or secretion of progesterone (Hoyer and Niswender, 1985). Thus, it is clearly evident that progesterone production in large cells is regulated in a different manner than in small cells.

Prostaglandin F_2 ($PGF_2\alpha$) and estradiol-17B (E_2) receptors have been identified in large luteal cells of the ewe (Fitz et al., 1982) and the cow (Koos and Hansel, 1981), and new information on effects of $PGF_2\alpha$ in small cell preparations suggest they may also possess the receptor for $PGF_2\alpha$ (Alila et al., 1988). Progesterone secretion by large, but not small, luteal cells was stimulated by PGE_2 and did not appear to involve activation of adenylate cyclase or increased intracellular cAMP concentrations (Fitz et al., 1984a). *In vitro* exposure of isolated large and small luteal cells to $PGF_2\alpha$ resulted in a dose-dependent reduction of progesterone accumulation in the media of large cells (Fitz et al., 1984b), whereas small cells did not appear to respond to $PGF_2\alpha$ (Fitz et al., 1984a). Glass et al. (1985) found that receptors for E_2 are fivefold more abundant in large than in small luteal cells. In view of the observations that large cells contain the majority of receptors for E_2 and $PGF_2\alpha$, it is possible that

regression of the CL and concomitant cessation of the secretion of progesterone at the end of the estrous cycle are brought about in part by the action of both $PGF_2\alpha$ and E_2 on the large luteal cells (Glass et al., 1984).

Small luteal cells are more numerous than large cells (Niswender et al., 1976), however, due to their size, large luteal cells account for approximately 30% of the CL on a volume basis compared to 16% for small cells. The remaining portion of the ovine CL consists primarily of three nonsteroidogenic cell types including fibroblasts, capillary endothelial cells and pericytes (O'Shea et al., 1979, 1980; Rodgers et al., 1984).

The cellular composition of CL undergoes dynamic changes and continual development throughout the entire estrous cycle in domestic ruminants. Composition of the ovine CL, as determined by morphometric analysis, revealed that the number of steriodogenic cells increased early in the cycle while the number of nonsteroidogenic cells tended to increase later in the cycle (Farin et al., 1986). In one study involving analysis of dissociated ovine luteal cells, Niswender et al. (1985) reported the total number of steroidogenic large and small cells increased approximately fourfold between days 4 and 8 of the estrous cycle, then declined through day 16. More specifically, Farin et al. (1986) found that the number of small luteal cells increased during the estrous cycle although the size of these cells remained

relatively constant. In contrast, large luteal cells, while remaining constant in number, continued to increase in size as the estrous cycle progressed. Farin et al. (1986) hypothesized that the observed increased size of large luteal cells may be indicative of further growth and development of these cells after luteinization.

Donaldson and Hansel (1965) first suggested that bovine small luteal cells differentiate into large luteal cells under the influence of LH during CL maturation. Using monoclonal antibodies against bovine granulosa and thecal specific antigens in an indirect immunofluorescence assay, Alila and Hansel (1984) observed that early in the bovine estrous cycle, from days 4 to 6, 77% of the large luteal cells bound the granulosa antibody, while 70% of the small cells bound the thecal antibody. However, as the estrous cycle progressed, there was a reduction in the proportion of large cells displaying fluorescence upon binding the granulosal cell antibody and a greater proportion of large cells binding the theca antibody. Small cells only exhibited a reaction with the theca antibody throughout the cycle. These changes in large cell antibody binding suggest that many large cells of the mature bovine CL are derived from transformed small cells (Alila and Hansel, 1984) and lend support to Donaldson and Hansel's earlier hypothesis. The observation by Cran (1983) that cells of the thecal layer of cystic bovine follicles acquired

characteristics ascribed to large cells after treatment with pregnant mare serum gonadotropin (PMSG) is also consistent with this hypothesis.

In ewes, Gamboni et al. (1984) observed a decrease in small cell numbers after treatment with human chorionic gonadotropin (hCG) on day 5 of the estrous cycle. Recently, Farin et al. (1988) reported that injection of ewes with pharmacological doses of LH every 6 hours from day 5 until day 10 or hCG injection on days 5 and 7.5 of the cycle led to an increase in large cell numbers with a concomitant reduction in small cells. Thus, in the ewe, the data are also consistent with the hypothesis that small luteal cells can further differentiate and give rise to large luteal cells.

HORMONES SYNTHESIZED BY THE CORPUS LUTEUM

Steroid Hormones

Progesterone

The primary function of the CL is generally considered to be the production of the steroid progesterone which is required for the maintenance of pregnancy in most mammalian species. During pregnancy, progesterone acts synergistically with estrogen in several physiologic functions including the growth of the uterus and mammary glands. In addition, progesterone inhibits uterine contractions and stimulates the endometrial glands to secrete histotroph which is

necessary for nourishment of the preimplantation blastocyst.

During the ovine estrous cycle, progesterone has been shown to perform important regulatory functions including the preconditioning of the central nervous system to the estrus-inducing properties of estrogen (Robinson, 1954) and controlling the time of ovulation; high levels of progesterone block the estrogen-induced LH surge and estrus (Scaramuzzi et al., 1971). Furthermore, Thomas et al. (1987) found that active immunization of ewes against progesterone caused a shortening of the interval between ovulations from 17-18 days in controls to between 6 and 10 days in immunized ewes with a corresponding reduction in the interval between LH surges. Progesterone-immunized ewes also were reported to have higher ovulation rates (1.72) than controls (1.25) and exhibited a reduced incidence of estrus (26% vs 95%). Moreover, progesterone has been shown to exert a negative feedback on LH in controlling the tonic secretion of this gonadotropin in the ewe (Goodman and Karsch, 1980) and to prime the ovine uterus for the release of prostaglandin (Scaramuzzi et al., 1977). These studies highlight the importance of progesterone in the control of estrus, ovulation, ovulation rate, luteal regression and the secretion of LH in the ewe. Effects of progesterone vary with the stage of the estrous cycle, in part because the responses are modified by the prior

and concomitant presence of estrogens. Muldoon (1980) found that estrogen induced progesterone receptors in the pituitary gland, hypothalamus, uterus and mammary glands.

Mechanisms involved in the synthesis and secretion of progesterone are complex, although this hormone is the first biologically active compound produced in the steroid biosynthetic pathway. The immediate precursor for all steroids is pregnenolone which is derived from cholesterol. Luteal tissue is thought to utilize cholesterol esters complexed with lipoproteins in serum as the major source of substrate for progesterone synthesis rather than cholesterol derived from *de novo* synthesis (Azhar and Menon, 1981; Schuler et al., 1981). The cholesterol ester carbon 3 (C-3) is cleaved by cholesterol esterase, and the free cholesterol is transported to the mitochondria (Flint et al., 1973). Within the mitochondria, cytochrome *P*-450 cholesterol side chain cleavage enzyme complex binds cholesterol, catalyzing hydroxylations at carbons 20 and 22, followed by cleavage at the C_{20} and C_{22} bond to yield pregnenolone and isocaproic acid (reveiwed by Savard, 1973). Conversion of cholesterol to pregnenolone has been shown to be rate-limiting in luteal steroidogenesis and is the point at which, or prior to which, stimulation of progesterone synthesis by tropic hormones is thought to occur (Hall and Koritz, 1964; Hall and Young, 1968). Pregnenolone diffuses from the mitochondria back to the

smooth endoplasmic reticulum where it is converted to progesterone by △5-3β-hydroxysteroid dehydrogenase △5,△4-isomerase complex (3B-HSD). While product inhibition has been reported for 3β-HSD (Caffrey et al., 1979), the conversion of pregnenolone to progesterone is not thought to be rate-limiting for steroidogenesis. Therefore, the rate of luteal steroidogenesis appears to be controlled by substrate availability, transport of cholesterol into the mitochondria or the activity of the side chain cleavage enzyme complex or combinations of these mechanisms.

Estrogens and Other Steroids

In addition to progesterone, the CL produces other steroids, the nature and quantity of which varies between mammalian species (Savard, 1973). In species studied, the bovine CL elaborates the simplest steroidal product which consists of mainly progesterone and some 20β-hydroxy-4-pregnen-3-one and pregnenolone (Mason et al., 1962). At the other end of the spectrum is the human CL which secretes progesterone as the major steroidal product but which is accompanied by 20α-hydroxy-4-pregnen-3-one, pregnenolone, 17B-hydroxyprogesterone, 4-androstenedione, estrone and estradiol-17β. Between these two extremes of steroidogenic capabilities lie the other species - the ewe (Kaltenbach et al., 1967), the sow (Cook et al., 1967), the mare (Mahajan and Samuels,

1963) and the rabbit (Telegdy and Savard, 1966). In addition to progesterone and its 20-dihydro product, the CL of these species produce 17α-hydroxyprogesterone, 4-androstenedione, but characteristically neither estrone nor estradiol-17β.

Although estrogens have the widest range of physiologic functions of all the steroids, this section will breifly mention only a handful of them. Estrogens are required for the induction of sexual receptivity and the psychologic manifestations of estrus or "heat". Furthermore, glandular duct growth of the uterine endometrium, histologic changes of the vaginal epithelium during the estrous cycle, and duct growth in the mammary gland during lactogenesis are attributable to estrogen. Other effects of estrogen include their ability to control the release of pituitary hormones, assist in the process of implantation, and potentiate the effects of oxytocin and prostaglandins on uterine contractions. The luteotropic and luteolytic effects of estrogen are discussed in subsequent sections of this review.

Protein Hormones

Oxytocin

Oxytocin is a neuroendocrine peptide that plays important roles in parturition and lactation by stimulating the activity of smooth muscle and myoepithelial cells, respectively. Although the

posterior pituitary is the major source of oxytocin released at parturition and in response to suckling, it is now well established that oxytocin is also located in the corpus luteum of several species. Corpora lutea of ewes (Wathes and Swann, 1982), cows (Fields et al., 1983) and goats (Homeida, 1986) were found to contain large quantities of oxytocin identical to that produced by the hypothalamus. In the ovine and bovine CL, as in the hypothalamo-hypophyseal system, oxytocin and neurophysin (a protein cosynthesized with oxytocin and thought to serve as a carrier) are produced from the same messenger ribonucleic acid (mRNA) and cleaved from the prohormone after translation (Ivell and Richter, 1984; Jones and Flint, 1986). Secretion by the ovine CL of equimolar quantities of oxytocin and neurophysin is consistent with this biosynthetic mechanism (Watkins et al., 1984).

In the ewe and cow, the capacity for luteal oxytocin synthesis first appears in granulosa cells of preovulatory follicles and immunohistochemical studies indicate that oxytocin and its associated neurophysin later reside in large luteal cells (Rodgers et al., 1983a; Sawyer et al., 1986, Fehr et al., 1987). More specifically, subcellular localization of oxytocin and neurophysin using an immunogold-electron microscope technique showed the presence of these peptides in the small dense granules of the ovine large luteal cells (Theodosis et al., 1986).

Apart from its established roles in lactation and labor, oxytocin is thought to be an important regulator of the estrous cycle. It has long been known that exogenous oxytocin can alter the duration of the bovine estrous cycle; it causes premature luteolysis in this species and shortens the cycle to approximately 8 days when administered daily during the first week of the cycle (Armstrong and Hansel, 1959). Treatment of ewes with exogenous oxytocin during the early luteal phase of the cycle failed to hasten luteolysis (Milne, 1963), however, immunization against oxytocin increased the length of the ovine estrous cycle (Sheldrick et al., 1980).

During the luteal phase of the estrous cycle in the ewe, oxytocin and oxytocin-associated neurophysin concentrations are increased in the systemic circulation (Sheldrick and Flint, 1981) with secretion of the hormone occurring in a pulsatile fashion during luteolysis (Fairclough et al., 1980; Flint and Sheldrick, 1983). Measurement of systemic oxytocin levels by radioimmunoassay during the ovine estrous cycle (Webb et al., 1981; Schams et al., 1982) consistently showed that oxytocin and progesterone concentrations increase and decrease in synchrony during the luteal phase. It was hypothesized that oxytocin found during the luteal phase of the estrous cycle and possibly during luteolysis originated from the CL. Support for this premise was

demonstrated in ewes by Flint and Sheldrick (1982) and Watkins et al. (1984) who found veno-arterial differences of oxytocin and oxytocin-associated neurophysin concentrations for ovaries containing CL but not those without CL. Walters et al. (1984) found higher levels of oxytocin in blood collected from the vena cava than from the jugular vein of cows during the luteal phase, lending further support to the hypothesis that elevated luteal-phase levels of oxytocin originated from the CL. These latter data were supported by observations that the luteal-phase oxytocin concentration was positively correlated with the number of CL present (Schams et al., 1982, 1983) and that plasma oxytocin concentrations could be reduced by cloprostenol-induced CL regression (Flint and Sheldrick, 1983).

A major function of this luteal oxytocin, at least in ewes, cows and goats, appears to be the stimulation of uterine secretion of $PGF_2\alpha$, which in turn acts on the CL to promote further secretion of oxytocin and initiate luteolysis (Flint and Sheldrick, 1986; Wathes et al., 1986b; Homeida and Khalafalla, 1987). Therefore, the two hormones comprise a positive feedback loop, each capable of stimulating secretion of the other. This is supported by the observation that coincident surges of the PGF_2 metabolite, 13,14-dihydro-15-keto-$PGF_2\alpha$ (PGFM) and oxytocin occur when hourly plasma samples are taken during luteolysis (Flint and Sheldrick, 1983). Further

support for oxytocin causing uterine $PGF_2\alpha$ release during luteolysis is provided by the fact that exogenous oxytocin can stimulate the release of uterine $PGF_2\alpha$, particularly at the end of the cycle when there is a large increase in the number of endometrial oxytocin receptors (Roberts et al., 1975, 1976; Roberts and McCracken, 1976). Thus, in domestic ruminants, the positive feedback loop between luteal oxytocin and uterine $PGF_2\alpha$ appears to exist to ensure the demise of the CL in the absence of an embryo. Further studies concerning the role of oxytocin and $PGF_2\alpha$ in luteolysis can be found in later sections of this review.

Relaxin

Relaxin is a polypeptide hormone found primarily in tissues of mammalian reproductive organs during pregnancy. Site(s) of production of this hormone vary among species and include the corpus luteum, ovarian follicles, placenta, uterus and endometrium (reviewed by Anderson, 1987). Little research has been conducted concerning the sources of relaxin in ewes. Wathes et al. (1986) found placentomes to be the major source of relaxin immunoreactivity using an antiserum to porcine relaxin to assay concentrations of the hormone in ovine CL, placentomes and intercotyledonary endometrium. Unlike other species such as the sow and rat, however, there was no correlation of relaxin concentrations in the

CL with stage of pregnancy. Most research has been conducted using the rat, pig and cow as models, therefore this section will focus on the sources and actions of relaxin in these three species.

Corpora lutea of sows, rats, and cows are a rich source of biologically active relaxin during late pregnancy. Relaxin is synthesized and stored in luteal tissue of these species throughout pregnancy and then is released shortly before parturition. The rise and fall in relaxin concentrations in CL parallel the accumulation and disappearance of cytoplasmic granules in luteal cells suggesting that the granules are a site of relaxin storage (Sherwood et al., 1980; Sherwood and Rutherford, 1981; Anderson, 1987).

Extensive experimental evidence indicates multifaceted biological actions of relaxin during pregnancy and parturition. One critical role relaxin plays is suppression of uterine motility during late pregnancy. Relaxin is important during the period of declining progesterone and increasing estrogen domination of the uterus near term for the prevention of premature parturition (Downing and Sherwood, 1985b). Recording of intrauterine pressure of late pregnant rats indicates that the frequency and amplitude of uterine contractions are reduced when serum relaxin levels are elevated (Cheah and Sherwood, 1981: Mercado-Simmen et al., 1982).

Another important biological action of relaxin is the remodeling of connective tissue in preparation for imminent parturition. Cervical distensibility, dilation and softening are critical for passage of the fetus at birth. In most species, the nonpregnant cervix is a fibrous elastic structure. In the ewe, relaxin transformed the dense collagen fiber bundles of the cervix to a loose network of fibers (Fosang et al., 1984). Administration of porcine relaxin in late pregnant beef heifers increased both pelvic area and cervical dilation (Musah et al., 1986), and caused premature parturition without problems of dystocia (Musah et al., 1987). Relaxin is also necessary for ensuring a normal duration of parturition and frequency of live births in pigs (Nara et al., 1982) and rats (Downing and Sherwood, 1985a).

Understanding of the endogenous regulation of relaxin secretion by other hormones is less clear. In rats, relaxin secretion throughout the second one-half of gestation requires the presence of the placentae (Goldsmith et al., 1981). There is a direct relationship between number of rat conceptuses and serum and ovarian relaxin levels (Golos and Sherwood, 1982). A placental luteotropic effect overrides the suppressive effect of the maternal pitutitary on relaxin secretion (Golos and Sherwood, 1984: Sherwood et al., 1986).

In the sow, Felder et al. (1986) found that the placenta, fetuses, or even the uterus are not required for relaxin production or the precisely timed surge release of relaxin that occurs 113 days after estrus or mating (gestation = 114 days). Thus, the aging CL may be preprogrammed to release relaxin as a result of an inherent life span defined through evolutionary development. Although these ephemeral structures are capable of an extended life span to 150 days in hysterectomized sows, the CL retain this precisely timed signal for relaxin release on day 113. On the other hand, the control of the precisely timed signal could derive from the central nervous system and pituitary gland (reviewed by Anderson, 1987).

LUTEOTROPINS CONTRIBUTING TO LUTEAL MAINTENANCE

Luteinizing Hormone

One of the early crucial questions in reproductive physiology concerned the role of the pituitary gland in regulating the formation and maintenance of the CL in ruminants. It was previously established by Melampy et al. (1964) that prolactin from the anterior pituitary was the luteotropin in the rat. Kaltenbach et al. (1968a) found that hypophysectomy of ewes on day 1 after ovulation resulted in failure of the CL to form, while hypophysectomy on day 5 resulted in regression of the partially formed CL by day 12 of the estrous cycle. According to Denamur et al. (1973), even removal of the uterus did not preclude impaired luteal formation and function in the hypophysectomized ewe. Thus it was concluded that pituitary support is necessary throughout the estrous cycle of the ewe for normal luteal formation and function.

In the search for the adenohypophyseal hormone(s) responsible for luteal maintenance, various studies were conducted in which gonadotropic hormones were administered to hypophysectomized and intact ewes. Kaltenbach et al. (1968b) found that constant infusions of crude pituitary preparations containing LH and follicle stimulating hormone (FSH) and pure LH preparations maintained luteal function in both pregnant

and nonpregnant hypophysectomized ewes. In contrast, FSH, prolactin, or estrogen alone showed no luteotropic properties in these animals. A year later Hixon and Clegg (1969) demonstrated that injection of LH or human chorionic gonadotropin (hCG) into ewes hypophysectomized during mid-cycle significantly increased both the luteal concentration of progesterone and secretion rate of this steroid.

Subsequent studies by Karsch et al. (1971) indicated that constant infusions of LH, but not prolactin, also prolonged the life span and function of the CL in intact, cycling ewes. Daily treatment of cycling ewes with antiserum to LH on day 2 through 6 of the estrous cycle resulted in premature luteal regression (Fuller and Hansel, 1970). Furthermore, LH stimulated progesterone synthesis when incubated *in vitro* with ovine luteal tissue (Kaltenbach et al., 1967; Simmons et al., 1976). These data indicate that LH is the hormone primarily responsible for ovine luteal function during the estrous cycle.

There is considerable evidence indicating that LH is also the primary luteotropin in the cow. Luteotropic properties of LH *in vivo* were first discovered by Simmons and Hansel (1964) in the cow. These researchers demonstrated the ability of this gonadotropin to overcome the inhibitory effects of injected oxytocin on luteal

life span during the bovine estrous cycle. Subsequently, Donaldson and Hansel (1965) found that a single injection of bovine LH on day 16 of the estrous cycle prolonged the duration of the cycle from an average of 20.0 to 36.4 days. In addition, Carlson et al. (1971) showed that injection of bovine LH significantly increased plasma progesterone concentrations in both hysterectomized and intact heifers. Other experiments have shown that addition of LH to incubating bovine CL significantly increased progesterone synthesis *in vitro* (Mason et al., 1962; Armstrong and Black, 1966; Moody and Hansel, 1969) and that anti-bovine LH depressed CL function *in vitro* and *in vivo* (Hansel and Seifart, 1967; Snook et al., 1969).

Mechanism of Action

Luteinizing hormone is the major hormonal regulator of progesterone synthesis in luteal cells and the mechanism of action has been shown to conform to the "second messenger" model (reviewed by Marsh, 1976). Luteotropic properties of LH are expressed via a cascade of biological events originating at the luteal plasma membrane. The initial event which sustains and(or) stimulates steroidogenesis in the luteal cell is the binding of LH to a specific receptor in the plasma membrane (Catt and Pierce, 1978). This binding promotes

an interaction with membrane-bound adenylate cyclase, a multi-subunit enzyme coupled to the LH receptor (Marsh, 1975). Adenylate cyclase converts adenosine triphosphate (ATP) to cAMP, the second messenger which is necessary for steroidogenesis.

Activation of Adenylate Cyclase

Adenylate cyclase, structured within the lipid framework of the luteal cell membrane, is composed of a catalytic unit (C) and a nucleotide regulatory component (called G-proteins or N-proteins) containing guanosine triphoshpate (GTP) binding sites. The C-subunit catalyzes the formation of cAMP from ATP and Mg^{2+} (Rodbell et al., 1980). The G-proteins are signal-transducing proteins responsible for mediating the effects of GTP and regulating the activity of C.

Two types of G-proteins have been distinguished functionally. One mediates stimulation (G_s), the other inhibition (G_i) of the adenylate cyclase activity by GTP (Hildebrandt et al., 1983). Both G-proteins have been isolated and purified, and while they have converse functions, they do have similar structures. G_s has been shown to have an oligomeric structure with 45,000, 35,000 and 10,000 Dalton subunits, while G_i has a similar structure with 41,000, 35,000 and 10,000 dalton subunits (reviewed by Gilman, 1984). Both G-proteins are composed

of an alpha, beta, and gamma subunit. Although the beta (35 K) and gamma (10 K) subunits of G_s and G_i are identical, the larger alpha subunits are heterogeneous with respect to their molecular weights. The alpha subunit is responsible for binding guanine nucleotides and is affected by bacterial toxins. The $alpha_s$ can be ADP-ribosylated by cholera toxin, while the $alpha_i$ is ADP-ribosylated by pertussis toxin. The ADP-ribosylation of $alpha_s$ by cholera toxin results in the inhibition of GTPase activity of G_s, whereas the ADP-ribosylation of $alpha_i$ by pertussis toxin blocks the action of G_i. Such ADP-ribosylation results in characteristic modifications of function of each G-protein.

The stimulatory protein, G_s, is reported to have a dual effect in the adenylate cyclase system, enhancing enzymatic activity and modulating the affinity of receptor for the hormone (Limbird, 1981; Birnbaumer et al., 1985). These activities were found to require binding of GTP, or an analog, to G_s and were manifested as an enhancement of hormonal stimulation by GTP and a reduction in hormone binding in the presence of the nucleotide (Limbird, 1981; Birnbaumer et al., 1985). As a result of intrinsic cycling, the rate of which is increased by the presence of a stimulatory hormone, GTPase activity inherent to G_s causes the hydrolysis of GTP to GDP. This results in the inactivation of G_s

unless dissociation of the GDP and further GTP binding occurs (Jakobs et al., 1982). When inhibitory hormones bind to their receptor, G_i is activated which acts to inhibit adenylate cyclase activity. Inhibition of adenylate cyclase by G_i appears to prevent the alpha subunit of G_s from complexing with C, but may also involve a direct action by G_i (Gilman, 1984).

Activation of cAMP Dependent Protein Kinases

The actions of cAMP appear to be mediated by a cAMP-dependent protein kinase; a tetramer composed of two regulatory and two catalytic subunits. The two regulatory subunits contain two cAMP binding sites each, and are linked together by disulfide bonds. In the absence of cAMP, the protein kinase complex is inactive. This enzyme is activated by cAMP binding to the regulatory subunits (reviewed by Flockhart and Corbin, 1982), which promotes the dissociation of the catalytic subunits from the regulatory subunits. Catalytic subunits, freed from inhibition by the binding of cAMP, utilize Mg^{2+} and ATP to phosphorylate serine and threonine residues of various endogenous protein substrates, which results in modified activity of these biological regulators (Hunzicker-Dunn and Birnbaumer, 1985). Ultimately, a biological response is triggered. To terminate the actions of these second messengers, cAMP

dissociates from the cAMP-dependent kinase's regulatory subunit (Krebs and Beavo, 1979) and cAMP is degraded to 5'-AMP by a cAMP-phosphodiesterase (Marsh, 1975).

Luteal Steroidogenesis

Ling and Marsh (1977) found that the activity of protein kinase is enhanced in steroidogenic tissue at concentrations of LH that do not result in measurable increases in cAMP. The increase observed in protein kinase activity and progesterone secretion were highly correlated. Thus, it seems reasonable to assume that increased activity of protein kinase due to elevated intracellular levels of cAMP is involved in the stimulation of steroidogenesis by LH (Niswender and Nett, 1988).

Luteinizing hormone may stimulate the synthesis of progesterone via a number of potential mechanisms involving increased protein kinase activity. These include initiation of protein synthesis (Reel and Gorski, 1968; Azhar and Menon, 1975), including a cholesterol binding protein (Simpson et al., 1978) and stimulation of two enzymes involved in steroid biosynthesis, cholesterol esterase (Caffrey et al., 1979) and cholesterol side chain cleaveage enzyme, the rate-limiting factor in steroidogenesis (Caron et al., 1975; Neymark et al., 1984). Furthermore, enhanced protein kinase activity may

result in increased substrate available for steroid biosythesis due to activation of microfilaments involved in lipoprotein uptake (Niswender and Nett, 1988). Microfilaments may also play a role in the transport of pregnenolone, the immediate precursor of progesterone, out of the mitochondria (Hall, 1985). Additionally, reports have demonstrated significant increases in phoshpolipid content in rat Leydig cells in response to 8-bromo-cAMP and LH (Lowitt et al., 1982). It is not necessary that the actions of LH be limited to one of these mechanisms, moreover, there is general consensus that LH acts via several mechanisms to stimulate progesterone secretion.

Phospholipase C Second Messenger System

Although most attention has focused on the ability of LH to trigger the action of the cAMP second messenger system, there is evidence to suggest that LH may also stimulate progesterone synthesis via another second messenger system. Activation of a different set of specific cell surface receptors stimulates the inositol phospholipid-phospholipase C transmembrane signalling system. This system depends on the hydrolysis of membrane phosphotidylinositol 4,5-bisphosphate to yield inositol trisphosphate and diacylglycerol, which serve as second messengers (reviewed by Berridge, 1984).

Diacylglycerol acts to activate protein kinase C (Nishizuka, 1986) while inositol trisphosphate mobilizes intracellular calcium (Berridge, 1987), which is required for maximum stimulation of protein kinase C. This signalling pathway has been recently described in luteal cells of several domestic species. Luteinizing hormone and hCG have been observed to stimulate inositol phospholipid hydrolysis in addition to increasing intracellular cAMP in luteal cells from cows (Davis et al., 1981; Davis et al., 1987) and sows (Allen et al., 1988). Strauss (1982) proposed that LH-stimulated alteration of phospholipids may cause compositional changes in mitochrondrial membranes leading to enhanced conversion of pregnenolone to progesterone. These studies indicate that gonadotropins are capable of activating at least two transmembrane signalling systems. However, more research is required to determine the precise role of the inositol phospholipid-phospholipase C signalling system in mediating the actions of hormones in the ovary.

Structural Properties of LH and its Receptor

Luteinizing hormone of anterior pituitary origin and hCG of placental origin are both dimeric glycoproteins composed of two noncovalently linked alpha and beta subunits. Although both gonadotropins have identical

alpha subunits, the beta subunits share only 82% amino acid sequence homology, thus conferring biological specificity (reviewed by Pierce and Parsons, 1981). The alpha subunits of both glycoproteins are glycosylated and are cross-linked by disulfide bridges (Strickland et al., 1985).

Due to similarities in structure and function, LH and hCG bind to the same luteal plasma membrane receptor site. The presence of specific receptors for LH and hCG has been demonstrated in ewes (Diekman et al., 1978a), cows (Rao, 1974), rats (Lee and Ryan, 1972) and women (Lee et al., 1973).

During the past 15 years, different techniques have been used to examine the structure of the LH/hCG receptor, referred to as the LH receptor in this discussion. Although researchers tend to agree that the LH receptor is an oligomeric structure, there is no consensus on the number of subunits, their molecular weights, or on the nature of the bonds that hold these subunits together. The conflicting models proposed for the structure of the LH receptor can be divided into two major groups.

Using techniques such as chemical cross-linking (Metsikko, 1984; Kellokumpa and Rajaniemi, 1985), affinity purification procedures (Aubry et al., 1982; Metsikko, 1984), or photoaffinity labeling (Rapoport et

al., 1984), most investigators have agreed that the LH receptor is a single noncovalently bonded oligomer composed of one subunit of M_r = 79,000 to 100,000. A similar model was proposed by Dattatryamurty et al. (1983) after a comprehensive study involving purification of large quantities of bovine CL, resulting in high yield, purity and stability of the LH receptor. These researchers concluded that the LH receptor was an oligomeric glycoprotein existing as a M_r = 280,000 species composed of two identical subunits which were in turn composed of two disulfide-bonded chains of M_r = 85,000 and M_r = 38,000. In a more recent study, Ascoli and Segaloff (1986), used ^{125}I-labelled derivatives of hCG and ovine LH cross-linked to the LH receptor of porcine granulosa cells and found the receptor to consist of two disulfide-bonded subunits of M_r = 23,000 and 83,000 with LH only binding to the latter subunit.

The second set of LH receptor models is based primarily on the work of two groups of investigators and is suggestive of a more complex structure involving four separate subunits. Using photoaffinity labeling (Ji et al., 1981) or chemical cross-linking (Hwang and Menon, 1984), these investigators determined four different subunits joined by disulfide bonds (Ji et al., 1981) or noncovalent bonds (Hwang and Menon, 1984).

In a recent review (Ascoli and Segaloff, 1989) argue that the evidence supporting a LH receptor composed of a single polypeptide is stronger that the evidence indicating that the receptor is a more complex structure composed of several subunits. Clearly, however, this issue has not been resolved and will require further research into purification techniques, isolation and expression of a full length complementary DNA, and demonstration of LH/hCG binding to the receptor and activation of adenylate cyclase.

Luteal LH Receptors During the Estrous Cycle

Concentrations of luteal LH receptors change throughout the estrous cycle of the ewe. Diekman et al. (1978a) quantified the numbers of both occupied and unoccupied luteal LH receptors in crude plasma membrane preparations obtained from ewes on days 2 through 16 of the estrous cycle. These investigators observed that the number of occupied receptors increased 84% from days 2 to 10 of the cycle, remained high through day 14 and then declined dramatically by day 16. There were parallel changes in the number of unoccupied receptors. Although the total number of receptors increased 40-fold during development of the functional CL, less than 1% of the luteal receptors were occupied by LH on day 10 of the cycle when progesterone secretion reached maximum levels.

These data suggest that only a very small percentage of luteal LH receptors need to be occupied to promote maximal progesterone secretion. This is in agreement with earlier data collected by Mendelson et al. (1975), which indicated that only 1% of the 6000 receptor sites per Leydig cell needed to be occupied to elicit maximal secretion of testosterone *in vitro*.

In addition to the observed changes in LH receptor concentrations throughout the cycle, Diekman et al. (1978a) found that the number of occupied and unoccupied LH receptors were both highly correlated with the weight of the CL, serum progesterone levels, and luteal concentrations of progesterone. These investigators did not observe any change in affinity of the LH receptor throughout the estrous cycle. Diekman et al. (1978a) suggested that changes in LH receptor concentrations, and not serum LH, which is secreted at very low levels during the luteal phase of the cycle (Niswender et al., 1968), play a major role in the regulation of luteal progesterone secretion. In support of this concept, these researchers found that the association between progesterone secretion and LH receptor numbers continued through luteal regression with a parallel reduction in both progesterone and total and LH-occupied receptor numbers. A direct cause and effect relationship between the decline in luteal LH receptors and progesterone

secretion during luteolysis, however, does not appear valid in view of the fact that progesterone levels decreased 14.5 hours prior to any significant loss of either occupied or unoccupied LH receptors in CL on day of PGF_2 -treated ewes (Diekman et al., 1978b). Therefore, while increased LH receptors may appear to regulate luteal progesterone synthesis, a reduction in these receptors on day 16 of the cycle does not appear to be the initial step in luteal regression.

Maximal binding of hCG during the mid-luteal phase of the estrous cycle was also shown to occur in CL of the cow (Spicer et al., 1981), sow (Ziecik et al., 1980) and mare (Roser and Evans, 1983). In addition, capacity to bind hCG and the functional state of the human (Rao et al., 1977) and primate (Cameron and Stouffer, 1982) CL are correlated. Thus, for a number of species it is clear that progesterone secretion is maximal when the number of luteal LH receptors is highest, but LH levels are low throughout most of the cycle.

Homologous Regulation of Receptor Turnover

An interesting aspect of the biological effect of LH (or hCG) on luteal cell function is the ability of this gonadotropin to control concentrations of its own receptors. This phenomenon of homologous receptor regulation, termed down-regulation, is common among

protein hormones and has been observed in a large number of target tissues. A dramatic reduction in LH receptor numbers after exposure to high levels of hCG was first reported by Conti et al. (1976) in luteinized rat ovaries. Suter et al. (1980) provided interesting evidence for the temporal regulation of ovine luteal LH receptors following exogenous administration of a supraphysiological dose (1 mg) of oLH to ewes on day 10 of the estrous cycle. By 10 minutes post-injection, serum LH levels increased 1000-fold and were accompanied by a 260% increase in the total number of LH receptors. However, 2 hours later, LH receptor numbers returned to control levels, decreased subsequently to 63% of control levels by 24 hours, and then proceeded to return to control levels by 48 hours post-injection. The dramatic changes in total LH receptor numbers were not accompanied by a decline in serum progesterone which remained equal to or above that observed in control ewes. Maintenance of progesterone secretion may have been due to the fact that occupied LH receptor concentrations never declined below pre-treatment levels. These data are in agreement with Hsueh et al. (1976) and Sharpe (1977) who demonstrated that serum testosterone levels in rats were not altered after hCG-induced reductions in testicular LH receptors. In contrast to these data, down-regulation of LH receptors by exogenous hCG resulted in reduced

progesterone secretion for up to 4 days in luteinized rat ovaries (Harwood et al., 1978). Furthermore, injection of LH or hCG has been shown to cause desensitization of adenylate cyclase in rat and rabbit CL throughout pregnancy and pseudopregnancy (Hunzicker-Dunn and Birnbaumer, 1976). Conti et al. (1976) demonstrated that induction of this refractory or desensitized state in ovarian adenylate cyclase by gonadotropin resulted from the loss of specific hormone receptor sites. The down-regulation phenomenon has also been demonstrated *in vitro*, affording a good model to study its mechanism (Schwall and Erickson, 1983). Both LH and hCG induced down-regulation of LH receptors. Interestingly, activators of adenylate cyclase, inhibitors of cAMP degradation or analogs of cAMP also resulted in a net loss of LH receptors (Schwall and Erickson, 1983). These observations indicate that over-stimulation of the luteal cell, regardless of the nature of the stimulator, results in loss of LH receptors.

Although down-regulation of the LH receptor has been studied for over a decade, the exact mechanism of this process is still not entirely clear. The prevailing view regarding the fate of LH receptors is that both occupied and unoccupied receptors are internalized into the luteal cell by endocytosis. Support for the concept of internalization comes from observations by Conn et al.

(1978) that several cell types internalize radioiodinated hCG. Once inside the luteal cell, the receptor-bound radioiodinated hCG appears to be degraded by lysosomal enzymes because when radioiodinated hCG was administered, iodotyrosine was the major radioactive product (Ascoli and Puett, 1978). Although the fate of the internalized receptor remains equivocal, it is suggested that once the internalized hormone and receptor separate, the receptor is recycled back to the luteal plasma membrane (Suter and Niswender, 1983). In support of the recycling hypothesis, specific, high-affinity LH receptors were identified in rough endoplasmic reticulum, Golgi, nuclear and lysosomal membranes from bovine CL (Rao et al., 1981; 1983). The binding characteristics of all but the nuclear and lysosomal LH receptors were similar to plasma membrane receptors; therefore, if the recycling hypothesis is correct, processing of the receptors beyond the lysosomes should restore binding properties to those observed in receptors prior to internalization.

Prolactin

Prolactin is a hormone secreted by the anterior pituitary gland whose primary action involves the development of the mammary gland and production of milk. In some species, prolactin also has a role as a component of the luteotropic complex. In the rat for example,

Major et al. (1967) found that LH enhanced the rate of progesterone biosynthesis both *in vivo* and *in vitro*. Prolactin, on the other hand, was reported to have little effect on steroidogenic rate (Huang and Pearlman, 1962) but was able to maintain function and structure of the CL in hypophysectomized rats (Macdonald et al., 1970). Cervical stimulation of the rat, either by mating or by other methods, was demonstrated to trigger daily surges of prolactin (Smith and Neill, 1976) which maintained CL function until day 8 or 9 of pregnancy or pseudopregnancy. However, a luteotropic complex of both prolactin and gonadotropins appeared to be necessary to optimally maintain pregnancy in the rat (Ahmad et al., 1969). In the mouse and hamster, prolactin and small quantities of LH are required to maintain the biochemical and morphological integrity of the CL (Greenwald and Rothchild, 1968). Prolactin also has been demonstrated by Grinwich et al. (1976) to play an essential role in maintaining concentrations of luteal membrane LH receptors.

As a result of early experiments with rodents, it was originally believed that prolactin was also a primary luteotropin in ruminants. Before the availability of highly purified hormones, it was reported that prolactin supported luteal function in hypophysectomized ewes (Hixon and Clegg, 1969; Schroff et al., 1971). However,

administration of 2-bromo-αergocriptine, (a potent inhibitor of prolactin secretion), to ewes suppressed systemic prolactin concentrations by more than 95% but failed to affect circulating progesterone levels or cycle length (Niswender, 1974). Continuous infusion of prolactin did not prevent luteal regression in hypophysectomized ewes during the cycle or early pregnancy (Kaltenbach et al., 1968a,b). Furthermore, continuous infusion of prolactin into intact ewes either systemically (Karsch et al., 1971a,b) or into the ovarian artery (McCracken et al., 1971) had no effect on luteal function during the estrous cycle.

In cows, prolactin did not alter the length of the estrous cycle (Smith et al., 1957) or the synthesis of progesterone (Hansel, 1966). Furthermore, Hoffman et al. (1974) demonstrated that treatment with prolactin antiserum or 2-bromo-αerogcriptine did not alter serum progesterone levels or estrous cycle length. It is concluded that LH is the dominant luteotropic factor in both the cow and ewe while prolactin has little or no luteotropic activity and is not necessary for normal luteal function in these species.

Estrogen

There is evidence that estrogen has luteotropic properties in several species of mammals. In the rabbit,

estradiol-17β is the essential luteotropic hormone, because maintenance of the CL after hypophysectomy is dependent upon treatment with estradiol-17β (Bill and Keyes, 1983). The importance of endogenous estrogen of follicular origin in the luteotropic process was highlighted by experiments in which CL of the rabbit regressed after destruction of follicles by x-irradiation; however, with estrogen treatment the CL were maintained and produced progesterone (Keyes and Nalbandov, 1967; Keyes and Armstrong, 1968). The fact that the rabbit CL is a target organ for estrogen was confirmed by the identification of estrogen receptors in luteal tissue (Lee et al., 1971; Scott and Rennie, 1971).

Gardner et al. (1963) demonstrated that daily estrogen injections into gilts beginning on or before day 11 of the estrous cycle prolonged the life span of the CL. Concurrent administration of both LH and estrogen to hypophysectomized sows beginning on day 12 of the cycle prolonged CL function to day 20 (Anderson et al., 1967). However, exogenous estrogen alone failed to prolong CL life span in hypophysectomized sows and hypophysial stalk-transected gilts suggesting pituitary gonadotropin involvement in porcine luteal maintenance. Further, Anderson et al. (1967) found that treatment of hypophysectomized and hysterectomized sows with LH and no estrogen did not maintain the CL. Maintenance of the CL

in the rat is also dependent on both the actions of estradiol-17β and the anterior pituitary gland hormones (Gibori et al., 1978).

In the ewe, exogenous estradiol-17β can either prolong the life of the CL or promote luteolysis depending upon the time of the estrous cycle when it is administered. Denamur et al. (1970) were able to prolong CL life span with twice-daily injections of estradiol benzoate starting on day 3 of the cycle; however, injections on day 11 and 12 of the cycle were luteolytic, hastening regression of the CL (Stormshak et al., 1969). Removal of endogenous estradiol-17β by destruction of the ovarian follicles resulted in prolonged maintenance of the ovine CL (Karsch et al., 1970). Estrogen is not considered luteotropic in the cow and if exogenous estradiol-17β is administered on days 5 to 7 postestrus, luteolysis and precocious estrus result (Brunner et al., 1969).

Embryonic Luteotropins

The CL is necessary for maintenance of pregnancy for varying periods of time in different species. In the ewe, the CL must by present through approximately the first 50 days of gestation (Casida and Warwick, 1945) after which time the placenta takes over the role of providing progestational support (Ricketts and Flint,

1980). The cow, however, relies on the CL for progesterone throughout most of gestation (Estergreen et al., 1967). If fertilization does not occur in the ewe, the CL begins to regress about 13 days after estrus (Karsch et al., 1980) as a result of uterine release of a luteolysin, PGF_2^{α}, that reaches the ovary via the venoarterial pathway (Goding et al., 1972; Ginther, 1974). The presence of viable conceptuses within the uterus prevents CL regression. This phenonemon constitutes the so-called maternal recognition of pregnancy and is the result of secretions of antiluteolytic and/or luteotropic substances from the conceptus. Embryo transfer experiments indicated that a conceptus must be present in the ovine uterus by day 12 of pregnancy for luteolysis to be halted and pregnancy maintained (Moor and Rowson, 1966a). It was also shown that homogenates of conceptuses from days 14 to 15 of pregnancy maintained luteal function when infused into the uterus of nonpregnant, day 12 recipient ewes.

The precise nature of the conceptus signal(s), which occur during the 'critical period' to ensure luteal maintenance, have not yet been fully elucidated. One line of evidence points to prostaglandins of the E series (PGE_1 and PGE_2) as likely candidates acting as luteotropic factors or antiluteolysins during the maternal recognition of pregnancy in the ewe. Presence

of PGE_2 in the uterine endometrium during early pregnancy was initially reported by Wilson et al. (1972a) and a decade later LaCroix and Kann (1982) showed that PGE_2 is also secreted by the ovine conceptus. Silvia et al. (1984) observed that uterine secretion of PGE_2 is higher in pregnant than nonpregnant ewes. In addition, on days 14 to 16 of pregnancy, the ovine conceptus was found to contribute to PGE_2 production (LaCroix and Kann, 1982; Hyland et al., 1982). Chronic intrauterine administration of PGE_2 blocked natural luteolysis in ewes for two days (Pratt et al., 1979) and prevented estradiol-induced luteal regression (Colcord et al., 1978). In a later study, Magness et al. (1981) found that luteolysis was prevented in nonpregnant ewes by PGE_2 only when infused chronically into the uterine horn adjacent to an ovary bearing CL but not when infused into the contralateral uterine horn. In experiments where PGE_2 and $PGF_2\alpha$ were administered simultaneously, it was found that PGE_2 prevented $PGF_2\alpha$-induced reductions in progesterone secretion (Henderson et al., 1977) and luteal weight (Mapletoft et al., 1977). Furthermore, secretion of PGE_2 by endometrial tissue *in vitro* is greater in pregnant than in nonpregnant ewes (Marcus, 1981; LaCroix and Kann, 1982). These results suggest that both uterine and conceptus PGE_2 are luteotropic and

may contribute to extended function of the CL during the critical period in the ewe.

A second luteotropic/antiluteolytic factor of pregnancy that has claimed much attention in recent years is ovine trophoblast protein-1 (oTP-1; Godkin et al., 1982), an acidic protein of trophoblastic origin (Godkin et al., 1984a,b). Ovine trophoblast protein-1 shares considerable homology with bovine trophoblast-1 (bTP-1; Bartol et al., 1985) and with a class of molecules called alpha-interferons, which are involved in immune responses (Imakawa et al., 1987). The predominant secretion of oTP-1 was found to occur transiently between days 13 and 21 of pregnancy, after which time it was no longer detected (Godkin et al., 1982). To test the ability of oTP-1 to extend CL life span, Godkin et al. (1984b) introduced proteins released by cultured day 15 to 16 conceptuses into the uterine lumen of cyclic ewes late in the luteal phase of the estrous cycle. These investigators found that all ewes receiving the conceptus protein had functional CL until at least day 25, while one ewe continued to secrete progesterone until day 52. The mechanism whereby oTP-1 exerts its effects appears to be at the level of the maternal uterine endometrium (Godkin et al., 1984b). It is reported that oTP-1 acts in an antiluteolytic manner by altering protein (Godkin et al., 1984b) and prostaglandin metabolism (Vallet et

al., 1987; Salamonsen et al., 1988). The role of oTP-1 as an antiluteolysin rather than a luteotropin is supported by the observation that oTP-1 failed to displace both prolactin and hCG from their respective receptors, and it was unable to stimulate progesterone secretion from dispersed ovine luteal cells (Godkin et al., 1984a). Further support was provided by Fincher et al. (1986) who demonstrated that daily uterine infusion of day 16 conceptus secretory proteins into nonpregnant ewes treated with either E_2 or oxytocin suppressed release of PGF_2^{α}.

In summary, these studies suggest that alpha-interferon-like molecules produced by the conceptus (oTP-1), and PGE_2 produced by the conceptus and endometrium, act in a luteotropic and(or) an antiluteolytic manner to regulate the functional life span of the CL in the pregnant ewe.

REGRESSION OF THE CORPUS LUTEUM

Utero-Ovarian Functional Interrelationships

The first evidence of uterine involvement in the luteolytic process was presented in 1923 by Loeb who showed that removal of the uterus prolonged the life span of the CL in guinea pigs. Maintenance of the CL after total hysterectomy in sheep and cows was later demonstrated by Wiltbank and Casida (1956). These early

studies suggested that regression of the CL during the estrous cycle requires a stimulus from the uterus. Evidence indicates that the effects of the uterus on luteal function are exerted locally. Unilateral hysterectomy of ewes (Inskeep and Butcher, 1966; Moor and Rowson, 1966b) and cows (Ginther et al., 1967) resulted in normal luteal regression when the CL was in the ovary ipsilateral to the intact uterine horn but prevented regression of CL in the contralateral ovary. Thus, removal of the ovary from the local luteolytic factor emanating from the uterus prolongs the life span of the CL of these ruminants and high serum progesterone levels are maintained (McCracken et al., 1971; Hixon and Hansel, 1974). The presence of the uterus is not required for luteolysis in women and nonhuman primates and it has been suggested that a luteolysin is produced locally by the ovary in these species (Auletta et al., 1984).

Action of Prostaglandins

Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) has been identified as the luteolytic hormone of ewes (Thorburn and Nicol, 1971; Goding et al., 1972; McCracken et al., 1972) and cows (Liehr et al., 1972; Lamond et al., 1973; Thatcher and Chenault, 1976). Prostaglandin $F_2\alpha$ has been isolated from uterine endometrial tissue and has been shown to reach maximal concentrations during the period of luteal

regression in cows (Shemesh and Hansel, 1975) and ewes (Wilson et al., 1972b). In support of the premise that $PGF_2\alpha$ is the endogenous luteolysin, researchers have demonstrated that administration of this hormone to hysterectomized ewes (Bolt, 1973) and cows (LaVoie et al., 1975) caused luteal regression. Furthermore, natural luteolysis can be prevented by passive or active immunization of ewes and cows against $PGF_2\alpha$ (Scaramuzzi and Baird, 1976; Fairclough et al., 1976, 1981). Collectively, the above observations strongly suggest that $PGF_2\alpha$ of uterine origin has a definite physiological role in regulating luteal function in ruminants.

Near the end of the luteal phase of ruminant estrous cycle, $PGF_2\alpha$ is released from the uterus as a series of pulses (Thorburn et al., 1973; Barcikowski et al., 1974; Fairclough et al., 1980) and reaches the ovary via a countercurrent transfer of $PGF_2\alpha$ through the wall of the utero-ovarian vein into the ovarian artery (McCracken et al., 1972; Ginther, 1974; Hansel, 1975). Such a mechanism permits a small amount of $PGF_2\alpha$ (approximately 1%) to reach the ovary directly; otherwise, it would be rapidly degraded in the pulmonary vascular bed (Piper et al., 1970). New evidence shows that in the ewe, uterine $PGF_2\alpha$ can also be transferred locally from uterine lymphatic vessels to the adjacent ovary and ovarian vein (Heap et al., 1985). Measurement

of $PGF_2\alpha$ or its major circulating metabolite, 13,14-dihydro-15-keto-$PGF_2\alpha$ (PGFM) in peripheral or uteroovarian venous blood in ewes (Thornburn et al., 1973; Peterson et al., 1976) and cows (Fairclough and Payne, 1975) has revealed pulses of secretion during luteolysis, with a concomitant decrease in circulating progesterone. Intraovarian administration of exogenous $PGF_2\alpha$ to ewes as a series of discrete pulses was found to be more effective in causing luteolysis than a constant infusion (Schramm et al., 1983), suggesting a specific pulsatile pattern of $PGF_2\alpha$ release from the uterus is advantageous for luteolysis in this species.

Despite intense investigations, the precise mechanism by which $PGF_2\alpha$ results in luteolysis in the ewe remains unknown, although the luteolytic effects of $PGF_2\alpha$ have been ascribed in part to changes in blood flow (Nett et al., 1976). Originally, the luteolytic action of $PGF_2\alpha$ was thought to involve a reduction in blood flow to the ovary due to the known vasoconstrictive properties of $PGF_2\alpha$ (Pharriss et al., 1970). Although high doses of $PGF_2\alpha$ administered via the ovarian artery reduced ovarian blood flow to the autotransplanted ovary of ewes, lower doses caused luteal regession in the absence of any changes in blood flow (McCracken et al., 1971). In the intact ewe, Niswender et al. (1976) demonstrated that blood flow to the ovary bearing the CL was correlated

with systemic progesterone levels during the estrous cycle. Support for this study came with the finding that luteolysis, induced by $PGF_2\alpha$ injection, was accompanied by decreased blood flow to the luteal ovary as well as reduced systemic concentrations of progesterone (Nett et al., 1976). However, it has not unequivocally been proven that the effect of $PGF_2\alpha$ on ovarian blood flow is the cause and not the effect of luteal regession. Although the effect of $PGF_2\alpha$ on ovarian blood flow as a cause of luteolysis remains controversial, it does not preclude the possibility that ovarian $PGF_2\alpha$ could act by locally shunting blood away from the CL during regression (Einer-Jensen and McCracken, 1977; Janson et al., 1983).

The mechanism by which $PGF_2\alpha$ promotes luteal regression is more complex at the cellular level than can simply be explained by changes related to the vasoactive properties of this hormone. Because the ovine CL has abundant $PGF_2\alpha$ receptors (Powell et al., 1974), it is likely that $PGF_2\alpha$ effects on the CL are mediated at least in part by a direct interaction with luteal cells. A variety of changes in luteal tissues *in vitro* have been demonstrated after $PGF_2\alpha$-induced luteolysis, including effects upon LH-stimulated activity of adenylate cyclase (Evrard et al., 1978; Fletcher and Niswender, 1982), membrane fluidity (Riley and Carlson, 1985), and activities of various steroidogenic enzymes (Behrman et

al,. 1971; Caffrey et al., 1979; Torday et al., 1980). More recently, $PGF_2\alpha$ has been implicated in turnover of phosphatidylinositol and calcium mobilization in rat (Leung et al., 1986) and bovine (Davis et al., 1987) luteal cells. However, most mechanisms proposed for the action of $PGF_2\alpha$ on luteal cells involve interference with gonadotropin binding. *In vitro* investigation using dispersed cells or luteal minces from the rat have revealed that $PGF_2\alpha$ is a potent antagonist of the action of LH or hCG (Evrard et al., 1978; Behrman et al., 1979; Jordan, 1981). The rapid inhibitory effect of $PGF_2\alpha$ on rat luteal tissue leads to a block of LH-stimulated cAMP production (Lahav et al., 1976), via a mechanism not mediated through interference with LH binding (Thomas et al., 1978). Furthermore, $PGF_2\alpha$ blocks steroidogenic action of dbcAMP in rats (Jordan, 1981; Kenny and Robinson, 1986). Evidence relating to similar actions in ruminants is now becoming available, although it is less complete than in the rat. In the ewe, $PGF_2\alpha$ blocks adenylate cyclase and LH-stimulated progesterone synthesis when incubated with luteal slices (Evrard et al., 1978; Fletcher and Niswender, 1982). However, when isolated large and small luteal cells were incubated separately, treatment of the enriched large cell fraction with $PGF_2\alpha$ reduced progesterone secretion, but did not alter basal progesterone production by small cells

(Silvia et al., 1984). Because $PGF_2\alpha$ had no effect on LH-stimulated progesterone synthesis by small luteal cells but significantly inhibited that by small luteal cells contaminated by large cells, Rodgers et al. (1985) concluded that the inhibition of LH-stimulated progesterone synthesis by small cells is dependent on the presence of large luteal cells. It is thought that for $PGF_2\alpha$ to have a consistent antigonadotropic action, as in the whole cell preparations, the integrity of the luteal tissue must ensure some type of cell-to-cell communication (Auletta and Flint, 1988). Unfortunately, none of the effects of $PGF_2\alpha$ manifested by luteal tissue *in vitro* have been convincingly correlated with the rapid and marked degenerative events of luteolysis *in vivo*. It can be concluded from the above investigations that a clear-cut effect of $PGF_2\alpha$ in ovine luteal regression is still lacking.

Role of Steroid Hormones

Estrogen

Although the mechanisms controlling the synthesis and release of $PGF_2\alpha$ from the ruminant endometrium have yet to be fully elucidated, there is, however, evidence to suggest that at least three hormones are involved: estradiol, progesterone, and oxytocin. It has been clearly demonstrated that estradiol administered

systemically during the midluteal phase of the estrous cycle causes premature luteolysis in ewes (Stormshak et al., 1969; Hawk and Bolt, 1970) and cows (Brunner et al., 1969). On the other hand, destruction of ovarian follicles, the major source of estrogen by x-irradiation prolongs luteal function in ewes (Hixon et al., 1975) and cows (Villa-Godoy et al., 1981). Endogenous concentrations of estradiol are associated with maximal utero-ovarian levels of PGF_2^{α} during luteal regression in ewes (Baird et al., 1976; Scaramuzzi et al, 1977). Subsequent research has shown that the luteolytic effects of estradiol and PGF_2^{α} are additive in sheep and cows (Hixon et al., 1975, 1983) and the action of estradiol is not through inhibition of the steroidogenic stimulation of LH (Hixon et al., 1983). Finally, estradiol given systemically or locally causes uterine secretion of PGF_2^{α} in ewes (McCracken et al., 1971; Hixon and Flint, 1987) and cows (Hansel et al., 1973), with a subsequent decrease in progesterone concentrations and regression of the CL. These observations suggest that the interactions between estrogens and PGF_2^{α} play an important role during the luteolytic process in these ruminants.

Progesterone

Progesterone also appears to be involved in the physiological stimulus responsible for initiating the

synthesis and(or) release of $PGF_2\alpha$ from the ovine uterus, although the role of this steroid is less well defined than that of the estrogens. Scaramuzzi et al. (1977) demonstrated that exogenous progesterone administered to ovariectomized ewes for several days increases the uterine release of $PGF_2\alpha$. In a later study utilizing ewes with utero-ovarian autotransplants, McCracken (1980) showed that infusion of progesterone for 10 days beginning immediately post-estrus promotes a spontaneous release of $PGF_2\alpha$ following withdrawal of the steroid. Ottobre et al. (1980) suggested that a period of progestational influence regulates the timing of the initial small pulses of $PGF_2\alpha$ that precede luteolysis. These researchers demonstrated that the time of the initial release of $PGF_2\alpha$ was advanced from day 12 of the estrous cycle in controls to day 8 in ewes treated with progesterone on days 0 (day 0 = detected estrus) and day 1 of the cycle.

Role of Oxytocin

In addition to the ovarian steroids, there is evidence to suggest that endogenous oxytocin of luteal origin plays an essential role in regulating uterine secretion of $PGF_2\alpha$. Early evidence for a role of oxytocin in luteolysis was advanced by Sharma and Fitzpatrick (1974) and Mitchell et al. (1975) who

demonstrated that oxytocin administered to ewes caused the release of uterine prostaglandins. These studies stimulated Sheldrick et al. (1980) and Schams et al. (1983) to investigate whether oxytocin was involved in luteolysis by immunizing ewes against this hormone. They found that active and passive immunization against oxytocin prolonged the luteal phase of the estrous cycle, ultimately delaying luteal regression. In another study, Roberts et al. (1975) infused physiological quantities of oxytocin into the arterial supply of the ovine uterus and observed an increase in $PGF_2\alpha$ release; this response being present only late in the estrous cycle. A similar response was observed *in vitro* when Roberts et al. (1976) incubated ovine endometrial tissue with oxytocin and found maximal synthesis of $PGF_2\alpha$ on day 15 of the cycle. Oxytocin-induced $PGF_2\alpha$ secretion was presumed to occur through an oxytocin-receptor interaction because the highest concentrations of endometrial oxytocin receptors were observed during the late luteal phase of the cycle (Roberts et al., 1976).

More recently, McCracken et al. (1984) developed a hypothesis to explain the sequence of events that ultimately lead to luteal regression in the ewe. In brief, these investigators postulate that the decline in uterotropic actions of progesterone as the luteal phase progresses allows endogenous estradiol to stimulate

oxytocin receptor synthesis in the endometrium. Endogenous oxytocin, presumably of luteal origin, interacts with its receptor to cause secretion of PGF_2^{α} from the endometrium. Luteal regression is initiated following countercurrent transfer of PGF_2^{α} from the uterine vein to the ovarian artery. This $PGF2^{\alpha}$ then triggers further release of oxytocin from the CL and the resultant oxytocin binds to the endometrium stimulating further release of PGF_2^{α}. A positive feedback system is thus set up in which these two secretions are mutually reinforced. It is suggested that the latter release of luteal oxytocin causes the pulsatile secretion of PGF_2^{α} that occurs on days 14 and 15 of the estrous cycle. These late luteal phase pulses of endometrial PGF_2^{α} release occur every 6 hours and last for 1 hour periods. Oxytocin receptors may be desensitized for a period of time subsequent to oxytocin binding and this rest period between PGF_2^{α} pulses is hypothesized to allow time for estradiol to induce synthesis of new oxytocin receptors.

A slightly different and more elaborate role of oxytocin during luteal regression in ewes was proposed by Auletta and Flint (1988). These investigators hypothesize that the luteolytic action of PGF_2^{α} is initially exerted on the large luteal cells and can be extended to the small luteal cells through an inhibitory effect of PGF_2^{α} on local blood flow. Furthermore, they

suggest that an initial release of small quantities of uterine $PGF_2\alpha$ results in release of oxytocin and a reduction in progesterone production by the large luteal cells. The decrease in circulating progesterone causes an increase in uterine oxytocin receptors, which, interacting with secreted oxytocin, stimulates further release of $PGF_2\alpha$ in quantities sufficient to inhibit local ovarian blood flow and cause regression of the small cells. Such a mechanism would provide an explanation for the observation that the major release of $PGF_2\alpha$ from the uterus follows, rather than precedes, the commencement of luteolysis (Thorburn et al., 1973; Flint and Sheldrick, 1983) and for the coincident fall in progesterone and rise in uterine oxytocin receptors (Sheldrick and Flint, 1985). Because oxytocin is a vasoconstrictor in some organs, Auletta and Flint (1988) hypothesize that this peptide itself may exert an inhibitory effect on luteal blood flow following its release into the venous effluent from the large luteal cells. Both of these hypotheses are clearly open to further scrutiny, however, they do provide summaries of possible roles of oxytocin during the luteolytic process on which further research can be based.

Importance of Lipids in Luteal Fuction

ROLE OF THE PLASMA MEMBRANE IN LUTEAL FUNCTION

Structure and Fluidity of the Plasma Membrane

The plasma membrane is an important organelle in regulation of luteal cell function due to its ability to serve as a signal transducer. The luteal cell plasma membrane regulates transport of substrates and products between the cell and extracellular environment and acts as a framework of support for many proteins, including receptors for the hormones LH and $PGF_2\alpha$. Also found in this structure are receptor-activated enzymes, such as adenylate cyclase, which play important roles in the cellular response to hormone stimulation. Transmission of these hormone signals is initiated by specific hormone-receptor interactions across the plasma membrane of the luteal cell as has been discussed in a previous section of this thesis.

A characteristic of biological membrane function is fluidity, which refers to the degree of motion that membranous components can undergo in the course of functional interactions with each other (Singer and Nicolson, 1972). Membrane fluidity encompasses both rate and extent of movement of lipids as well as integral membrane proteins. Bulk fluidity characteristics of plasma membranes exist in two main phases called liquid-

crystalline phase, which represents the fluid state of membranes, and the gel phase, which represents a more solid or rigid state of the membrane (Thompson, 1980). Lipid molecules in cellular membranes are normally in a fluid state and diffuse readily in the plane of the bilayer.

With the exception of proteins, including those contributing to the structure of the receptors, the bulk of the plasma membrane is composed of phospholipids and their respective fatty acids (Singer and Nicolson, 1972). In mammalian ovaries, the major phospholipid species are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and the less abundant phospholipid components include phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SPH) and cardiolipin (Ansell et al., 1973). In terms of total percentage, Zelinski et al. (1988) found ovine luteal plasma membranes to contain approximately 49% PC, 33% PE, 10% SPH, 4% PS and 4% PI. These percentages of total phospholipids are similar to the composition of phospholipids found in bovine microsomal membranes (Goodsaid-Zalduondo et al., 1982) and CL homogenates (Scott et al., 1968). The fatty acid profile of ovine luteal tissue is made up of palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4) acid and minor quantities of

docosatetraenoic (22:4) and docosapentaenoic (22:5) acids (Zelinski et al., 1988).

Effect of Prostaglandins

In the last decade, researchers, employing various techniques, have shown that natural and induced luteolysis in the rat (Carlson et al., 1981; 1984) and cow (Carlson et al., 1982; Goodsaid-Zalduondo et al., 1982) is associated with a reduction in membrane fluidity. In a pair of studies using wide angle x-ray diffraction, Carlson et al. (1981, 1982) observed a phase transition in cellular membranes prepared from regressing rat and cow CL. In addition, these researchers reported that the amount of lipid in the gel phase increased as luteolysis advanced. In a subsequent study using fluorescence polarization, an optical technique of assessing membrane fluidity that relies upon the incorporation into membranes of fluorescent probe molecules, Carlson et al. (1984) again found a reduction in luteal plasma membrane fluidity during luteolysis in the rat. Using this same technique, Goodsaid-Zalduondo et al. (1982) found a decrease in the membrane fluidity of bovine luteal cells after prostaglandin treatment *in vivo*. The presence of gel phase lipid interferes with a number of important protein-mediated events in the membrane, since proteins are restricted from the gel

phase lipid (Shecter et al., 1974). Increased membrane viscosity can lead to a host of effects upon the membrane and ultimately the function of the cell. Some of these include alterations in receptor binding (Nunez and Glass, 1982) and membrane-bound enzyme activity, notably the adenylate cyclase system (Rimon et al., 1978). A reduction in membrane fluidity during luteolysis may explain the loss of gonadotropin binding and uncoupling of the LH-receptor complex to adenylate cyclase that is reported to occur at this time. According to the collision-coupling hypothesis proposed by Levitzki (1978), the plasma membrane lipid milieu provides the matrix through which the hormone-receptor complex must migrate in order to interact with the G_s prior to activation of the catalytic subunit of adenylate cyclase. Based upon the kinetics of adenylate cyclase activation, Levitzki (1978) provided evidence to suggest that the hormone-receptor complex must transiently encounter G_s within the plasma membrane. Therefore, from this model it can be predicted that alterations in membrane fluidity could affect hormone- stimulated adenylate cyclase activity.

Because lipids play a critical role in regulating membrane fluidity, several investigators have conducted studies to detect possible changes in the cholesterol to phospholipid ratio and fatty acid composition during

luteal regression. In rat luteal plasma membranes, the decrease in fluidity was not accompanied by major changes the cholesterol:phospholipid ratio or in the fatty acid composition (Carlson et al., 1981). Similarly, there was no change in the unsaturated to saturated fatty acid ratio or cholesterol to phospholipid ratio during luteolysis in the cow that might explain the appearance of gel phase lipid (Carlson et al., 1982). However, in another study in which the head group composition of phospholipid molecules in bovine CL was examined, Goodsaid-Zaluondo et al. (1982) observed a twofold increase in the sphingomylin content during luteolysis which may have promoted the observed reduction in membrane fluidity. In the ewe, luteolysis was associated with changes in arachidonic acid levels of phosphatidylcholine and phosphatidylinositol. The arachidonic acid content of phosphatidylcholine was increased while that of phosphatidylinositol was decreased (Zelinski et al., 1988). Furthermore, the docosatetraenoic acid (metabolite of arachidonate) content of phosphatidylinositol was also significantly higher in membranes of regressing ovine CL. Although these studies only focus on one stage of the life span of the CL, they do provide support for the idea that the luteal plasma membrane is an integral component in the regulation of CL function.

CYCLOPROPENOID FATTY ACIDS

Cyclopropenoid fatty acids (CPFA) are unique naturally occurring plant lipids containing a highly strained and reactive propene ring in the middle of their carbon chains. Two of the most abundant CPFA, the 18 carbon malvalate (2-octyl-1-cyclopropene-1-heptanoate) and 19 carbon sterculate (2-octyl-1-cyclopropene-1-octanoate) are found in seed lipids from plants of the order Malvales which includes cotton, kapok, okra, limes, durian, seeds of *Sterculia foetida*, and china chestnuts (Carter and Frampton, 1964; Greenberg and Harris, 1982.) Many of these plants are in the human diet or are consumed by animals in the human food chain. Cotton and kapok play especially important roles for man: cottonseed oil (*Gossypium hirsutum*) is present in many Western food preparations and livestock feed rations while kapok seed oil (*Eridendran anafractuosum*) is important in Asiatic diets. Both of these plants contain malvalic and sterculic acids with the latter reported to be the most biologically active CPFA. It has been suggested that CPFA may serve an antifungal role in plants because the concentrations of these fatty acids were within the range known to inhibit growth of some fungi (Schmid and Patterson, 1988).

Although little is known of the physiological or metabolic function of CPFA in plant species that synthesize them, it is well established that CPFA exert toxic and other adverse effects in a variety of animals. The earliest and most characteristic manifestation of CPFA toxicity documented was a prominent increase in saturated fatty acid content of tissue lipids with a concomitant decrease in the corresponding monoenes (reviewed by Matlock et al., 1985). Raju and Reiser (1967) and Johnson et al. (1967) demonstrated *in vivo* and *in vitro* that CPFA inhibit steroyl-CoA-$\triangle^9$-monodesaturase which synthesizes oleic from stearic acid. In a later study, Roehm et al. (1970) showed that CPFA fed to trout increased the ratios of stearic to oleic and palmitic to palmitoleic acid in liver triglycerides and phospholipids. In another study, Nixon et al. (1977) demonstrated that CPFA can be incorporated into phospholipids. After feeding rats a diet containing ^{14}C methyl sterculate, these investigators found significant quantities of labeled CPFA in the mitochondrial and microsomal fractions of hepatocytes by 4 hours after treatment. Thus, it is not only possible that CPFA alter the composition of phospholipids by increasing their concentration of saturated fatty acids, but these unique fatty acids also appear to become incorporated into

phospholipids themselves. In either case, the result may be a decrease in membrane fluidity.

In order to investigate the possibility that CPFA alter the composition of membrane proteins, Selivonchick et al. (1981) studied the microsomal protein components of hepatocytes of trout fed different levels of CPFA by use of SDS-polyacrylamide gel electrophoresis (SDS-PAGE). These investigators found that membrane fractions from CPFA-fed fish had reduced numbers of high molecular weight components and increased numbers of small molecular weight components. These data suggest CPFA can alter membrane protein composition by promoting degradation of microsomal proteins.

Dietary CPFA have also been reported to increase liver and blood cholesterol in rabbits (Ferguson et al., 1976) and mice (Matlock et al., 1985) and to induce aortic atherosclerosis in chickens and rabbits (Goodnight and Kemmerer, 1967; Ferguson et al., 1976). The reason for the cholesteremic activity of CPFA is unknown but it has been suggested to be an indirect consequence of $\triangle^9$-monodesaturase inhibition (Matlock et al., 1985). It is possible that CPFA inhibition of $\triangle^9$-monodesaturase leads to deficiencies or imbalances in the fatty acid profile of blood serum lipids that impair normal metabolism and clearance of serum cholesterol.

When fed as a protected lipid to goats and cows, CPFA increased the level of stearic relative to oleic acid in milk fat (Cook et al., 1976). Furthermore, milk fat from cows fed whole cottonseed (Smith et al., 1978) or cottonseed oil (Brown et al., 1962) had reduced levels of short-chain fatty acids.

Rainbow trout (*Salmo gairdneri*) have been shown to be extremely sensitive to the effects of CPFA. In early studies, it was discovered that CPFA could markedly stimulate both the incidence and growth of trout liver tumors induced by aflatoxin B_1, a potent carcinogen (Lee et al., 1968; Roehm et al., 1970). This cocarcinogenic response did not appear to result from a direct effect on DNA (Bailey et al., 1984), but rather from metabolic disturbances caused by CPFA. More specifically, CPFA fed to rainbow trout caused a marked reduction in enzyme activity of the mixed function oxidase system including benzopyrene hydroxylase, cytochrome *P*-450 and NADPH cytochrome *P*-450 reductase (Eisele et al., 1983). In a later study, it was shown that synthesis of acetyl-CoA carboxylase was reduced to 20% in rainbow trout raised on a diet containing CPFA compared to levels found in controls (Perdew et al., 1986).

Although the data is comparatively meager, ingestion of diets containing CPFA have been reported to interfere with reproduction in several species. Hens fed diets

containing sterculic acid had increased infertility and there was a high incidence of embryonic mortality in incubated eggs (McDonald and Shenstone, 1958). In rats, long-term feeding with up to 3% *Sterculia foetida* oil beginning at the weanling stage delayed the onset of puberty and thereafter prolonged the duration of the estrous cycle (Sheehan and Vavich, 1964). Furthermore, these investigators found that CPFA-fed rats had significantly smaller uteri than control animals. These latter data suggest that CPFA may have altered luteal function because progesterone produced by the CL is required for uterine development and normal cyclicity.

CYCLOPROPENOID FATTY ACID-INDUCED SUPPRESSION OF OVINE CORPUS LUTEUM FUNCTION

Introduction

Cyclopropenoid fatty acids (CPFA) are unique naturally occurring plant lipids containing a highly strained and reactive propene ring in the middle of their carbon chains. Two of the most abundant CPFA, the 18 carbon malvalate (2-octyl-1-cyclopropene-1-heptanoate) and 19 carbon sterculate (2-octyl-1-cyclopropene-1-octanoate) are found in seed lipids from plants of the order Malvales (Carter and Frampton, 1964; Greenberg and Harris, 1982.) Products of some of these plants, such as cottonseed oil or meal, are used in the human diet or are consumed by animals. Ingestion of diets containing sterculic acid (SA) delayed sexual maturity in poultry and rats (Sheehan and Vavich, 1964; Phelps et al., 1965).

Results of preliminary studies indicate that CPFA interfere with the formation and function of the developing corpus luteum in the ewe (Slayden and Stormshak, 1988). Injection of 750 ug of SA into the ovarian artery on day 2 of the estrous cycle suppressed serum progesterone concentrations and shortened the estrous cycle compared to the duration of cycles in oleic acid (OA)-treated ewes and DMSO-injected controls.

The present studies were conducted to (1) determine whether treatment of ewes with CPFA would cause regression of mature corpora lutea and if so, (2) whether CPFA-induced suppression of luteal function is due to an effect of these fatty acids proximal and(or) distal to adenylate cyclase.

Materials and Methods

ANIMALS AND SURGICAL TECHNIQUES

Thirty-three mature Western range ewes were monitored twice daily for estrous behavior by use of vasectomized rams. Ewes exhibiting at least one estrous cycle of normal duration were assigned randomly to one of three experiments. Ewes were fasted 24 h prior to surgery. On day 10 of the cycle, ewes were anesthetized by intravenous injection of sodium thiamylal (Biotal 2.5%) which was then maintained during midventral laparotomy by inhalation of halothane-oxygen. All surgical procedures were conducted under aseptic conditions.

CYCLOPROPENOID FATTY ACID PREPARATION

Purified preparations of SA and malvalic acid are not available commercially or from private sources. Therefore, a mixture of esterified fatty acids (methyl

esters) containing CPFA (sterculic acid 69%; malvalic, oleic, stearic, palmitic acids 31%) extracted from *Sterculia foetida* seeds was utilized. The extract was stored under N_2 at -20° C prior to dilution with dimethyl sulfoxide (DMSO). Cyclopropenoid fatty acid concentrations used are based on the sterculate (SA) fraction in the mixure because it has been reported to be the most biologically active CPFA (Pawlowski et al., 1985).

EXPERIMENT 1

This experiment was conducted to determine whether treatment of ewes with SA would interfere with luteal function and(or) cause regression of mature corpora lutea. Twelve ewes were assigned randomly in equal numbers to a control or treatment group. Reproductive organs were exposed by a midventral incision and the ovaries examined for the presence of corpora lutea. Ewes with corpora lutea in both ovaries were subjected to unilateral ovariectomy. However, if corpora lutea were present on one ovary only, the animal was allowed to remain intact. Treatment consisted of an injection of 1.09 mg of a fatty acid mixure (in 0.2 ml DMSO) containing the equivalent of 750 ug of SA into the artery supplying the ovary bearing corpora lutea. Control ewes were similarly injected with 1.09 mg oleic acid.

A jugular blood sample was collected prior to surgery, 12 h after surgery, and then daily until day 13 of the cycle or until the animal exhibited behavioral estrus, whichever came first. The resulting sera were stored frozen (-20^{o} C) until assayed for progesterone by use of radioimmunoassay.

EXPERIMENT 2

Experiment 2 was conducted to determine whether *in vitro* exposure of luteal tissue to SA would affect synthesis of progesterone during short-term incubation with ovine LH or dbcAMP. Six ewes or pairs of ewes were laparotomized and corpora lutea were removed, decapsulated, weighed and sliced (0.3 mm thickness) with a Harvard apparatus #140 tissue slicer. Luteal slices from each ewe or combined tissue from a pair of ewes was rinsed twice with Ham's F-12 medium, divided into eight aliquots (80-100 mg) and placed into 10 ml flasks containing 3.75 ml Ham's F-12 medium and 106 ng/ml (400 ng of *S. foetida* seed extract dissolved in 25 ul DMSO) of either SA (treatment; n = 4) or OA (control; n = 4). The flasks were incubated at 37^{o} C in a Dubnoff metabolic incubator under 95% O_2-5% CO_2 for 90 min.

After a triple rinse to remove the fatty acids, 3.75 ml of fresh medium were added to each pair of flasks plus the following additives: Flask 1, unincubated controls

(25 ul saline); Flask 2, incubated controls (25 ul saline); Flask 3, 40 ng ovine LH (oLH; in 25 ul saline; final concentration 10.67 ng/ml); Flask 4, 18.7 mg dbcAMP (in 25 ul saline; 10.64 mM final concentration). Flasks 2-4 were reincubated for 90 min as described above. Six milliliters of cold absolute ethanol were added to the SA and OA unincubated controls at the beginning of the second incubation and a similar quantity was added to the six remaining flasks at the end of incubation. The contents of each flask were transferred to vials along with two 3 ml rinses of each flask and the samples stored at -20^{o} C until assayed for progesterone by radioimmunoassay.

EXPERIMENT 3

Because LH is luteotropic in the ewe, this final experiment was conducted to determine whether sterculic acid impairment of luteal function involved a reduction in the number of luteal plasma membrane LH receptors. Eleven ewes were assigned to a control (n = 5) or treatment (n = 6) group and were laparotomized as described for Exp. 1. Treatment consisted of an injection of 750 ug of SA (1.09 mg total lipid) or 1.09 mg OA (in 0.2 ml DMSO) into the artery supplying the ovary bearing the greatest number of corpora lutea. Thirty hours after injection ewes were relaparotomized

and corpora lutea from the ovary receiving OA-injection and corpora lutea from both the SA-injected ovary and the contralateral ovary (negative controls; n = 5) were excised. Corpora lutea were trimmed of excess connective tissue, sliced in half, weighed and rapidly frozen in phosphate buffered saline (0.01 M; pH 7.0) 20% glycerol at -70° C and stored at this temperature until assayed for unoccupied LH receptors as described by Rodger and Stormshak (1986).

Tissue Preparation and Protein Determination

On the day of each assay, luteal tissue was sliced frozen and homogenized in 0.25 M sucrose, 25 mM Tris-HCl, 1 mM $CaCl_2$ (pH 7.4; 10 ml/g tissue) over ice using a 10-ml capacity Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was filtered over ice through two layers of cheesecloth and centrifuged at 800 x g at 4° C for 10 min. The nuclear pellet was discarded and the supernatant recentrifuged at 20,000 x g at 4° C for 20 min. The resulting supernatant was discarded and the membrane pellet resuspended in 0.05 M Tris-HCl (pH 7.4; 3 ml/g tissue). Ten, 15, and 20 ul fractions of luteal membrane preparation were assayed for protein (BSA Protein Assay Reagent; Pierce, Rockford, IL). Following protein determination, membrane preparations were diluted

in 25 mM Tris-HCl, 1 mM $CaCl_2$ (pH 7.4) to provide a final concentration of 1 ug protein/ul.

Radioiodination of hCG

Prior to iodination, highly purified hCG (CR-121; 13,450 IU/mg; supplied by the National Hormone and Pituitary Agency, NIADDK) was weighed and solubilized in 0.05 M phosphate buffer (pH 7.6) to a concentration of 5 ug hCG per 20 ul solution. Iodination was performed by adding 1 mCi ^{125}I and 10 ul chloramine-T (0.5 mg/ml) to the solubilized hCG and gently agitating the mixture for 3 min. The iodiation reaction was terminated with the addition of 10 ul sodium metabisulfite (1 mg/ml). Separation of labeled hormone from free iodine was performed by filtration through an anion exchange column consisting of a 3 ml plastic syringe packed with Analytical Grade Anion Exchange Resin (AG 2-X8, 100 - 200 mesh chloride form, Bio-Rad Laboratories, CA). The resin bed was equilibrated with 0.5 M sodium phosphate buffer (pH 7.6) and coated with 1.0 ml of 5% bovine serum albumin (BSA). To separate the labeled hormone from other components of the reaction mixture, it was layered on top of the resin bed, flushed through the column with 2 ml of 0.05 M sodium phosphate buffer (pH 7.5) and collected in a tube (12 x 75 mm) containing 1.0 ml of gelatin-phosphate buffered saline. Ten microliters were

counted to establish the concentration of purified labeled hCG.

Maximum Bindability

The proportion of radioactivity that represents biologically active hormone not damaged by the iodination procedure was determined by incubating a constant amount of ^{125}I-hCG (25,000 cpm/tube) with increasing amounts of luteal plasma membrane protein (0-150 ug/tube) in the presence or absence of 250 ng unlabeled hCG (Pregnyl; 1500 IU/mg; Organon Inc., West Orange, NJ) at 25^{o} C for 16 h. Quantity of specifically bound ^{125}I-hCG was calculated by subtracting nonspecific binding (membranes incubated with labeled hCG and Pregnyl) from the total binding (membranes incubated with labeled hCG alone) after separation of bound from free ^{125}I-hCG. Specific binding of labeled hormone was expressed as a percentage of the total cpm added and plotted as a function of increasing membrane protein as depicted in Figure 1. The active hormone fraction, which represented maximum binding, was 39% in the presence of 150 ug membrane protein. Nonspecific binding remained constant at 3.6% of the total radioactivity added at all membrane protein concentrations. Aliquots of 50 ug were routinely used in all subsequent assays. All calculations were corrected for maximum bindability during Scatchard (1949) analyses,

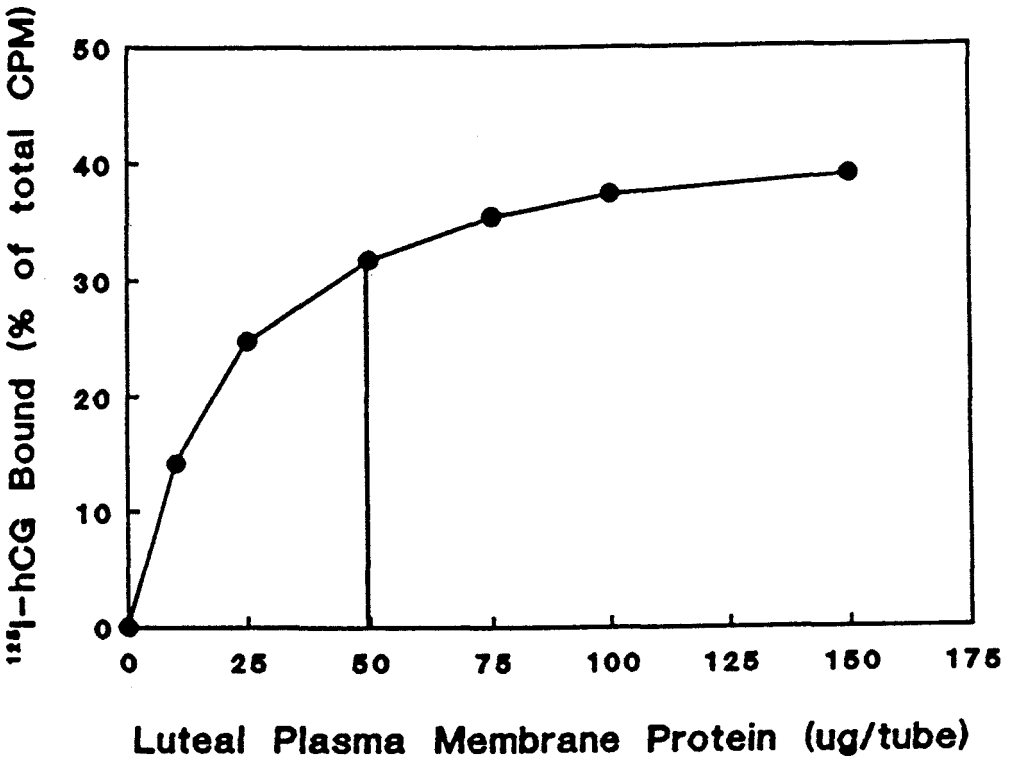

Figure 1. Maximum binding of ^{125}I-hCG was determined by incubating a constant amount of labeled hCG (25,000 cpm/tube) with increasing concentrations of membrane protein (0-150 ug protein/tube) in the presence or absence of 250 ng of Pregnyl. Maximum binding was 39% in the presence of 150 ug membrane protein.

because the ligand specifically bound to luteal tissue is believed to represent only intact hCG.

Specific Activity

The specific activity of the labeled hormone was determined in a self displacement radioligand-receptor assay (Ketelslegers et al., 1975). Increasing concentrations of unlabeled hCG (0.1 - 100 ng) were added to tubes containing a constant quantity of luteal plasma membrane (50 ug protein/tube) and ^{125}I-hCG (25,000 cpm/tube) to generate curve A (Figure 2). Curve B was obtained by adding increasing concentrations of ^{125}I-hCG (2.5×10^4 - 50×10^4 cpm/tube) to tubes containing a constant quantity of protein. Specific activity of the labeled hCG preparation, 35.5 uCi/ug, was calculated using a weight of 38,000 daltons for hCG, 37.5% counting efficiency and correction for maximum binding.

Measurement of hCG Binding

Quantity of labeled hCG needed to saturate all LH binding sites in 50 ug luteal plasma membrane protein was determined by the method of Diekman et al. (1978) with modifications. Saturation analysis was performed in 12 x 75 mm conical polystyrene tubes (Sarstedt, Princeton, NJ) using 50 ul membrane suspension (1 ug protein/ul, 25 mM Tris-HCl, 1 mM $CaCl_2$; pH 7.4) in triplicate from two

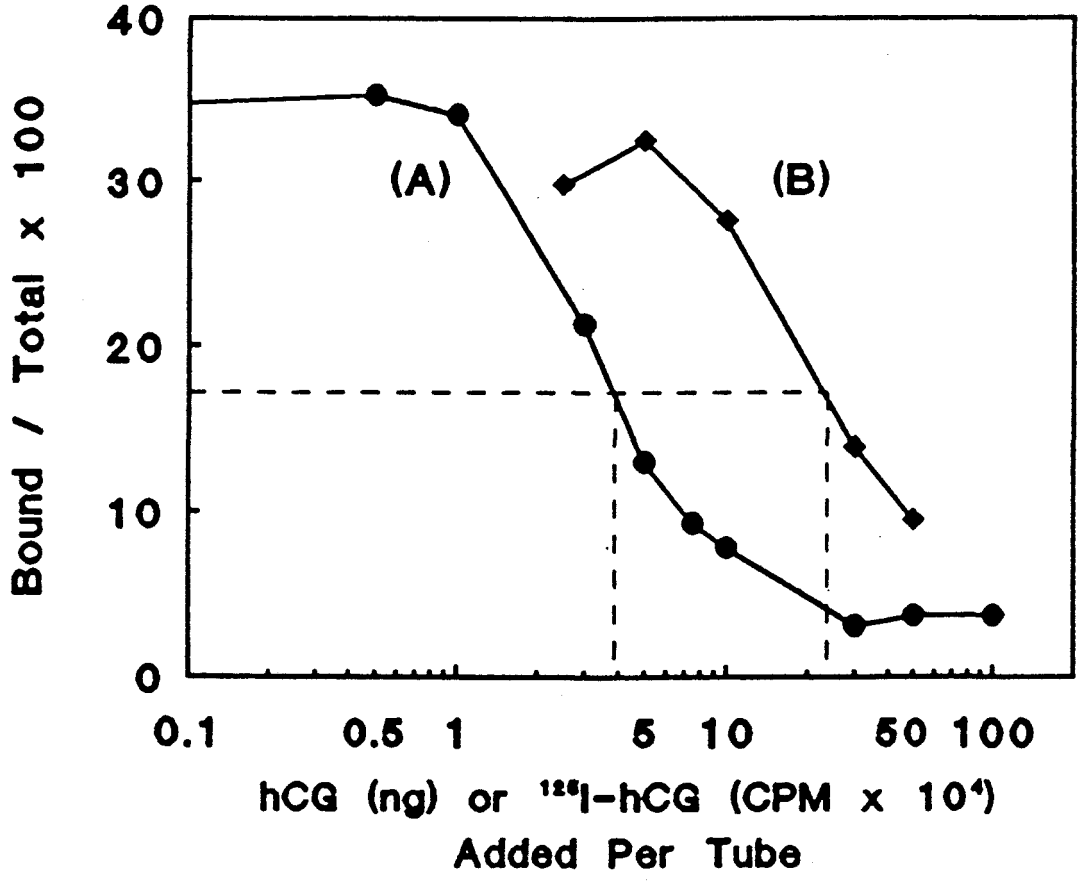

Figure 2. Specific activity of radioiodinated hCG was determined by self-displacement analysis. Curve A represents the percentage specific binding of 25,000 cpm labeled hCG/tube in the presence of increasing amounts of unlabeled hCG (Pregnyl). Curve B depicts total binding of increasing amounts of labeled hCG. Each tube contained 50 ug luteal plasma membrane protein. Specific activity was found to be 2.7 x 10^5 cpm/3.6 ng which converts to 35.1 uCi/ug when corrected for maximum binding and counting efficiency.

control and three treated ewes.

Increasing concentrations of ^{125}I-hCG (1 x 10^4 - 50 x 10^4 cpm/tube) plus Tris-HCl, $CaCl_2$ buffer or labeled hCG plus a 1000-fold excess of Pregnyl and buffer were added providing a final incubation volume of 0.5 ml. Following incubation for 16 h at 25° C, bound and free hormone were separated by precipitation of the bound fraction with 20% polyethyleneglycol (PEG; MW 7000-9000 , J.T. Baker Chemicals, NJ) as described by Bramley and Ryan (1978). Briefly, 0.5 ml of cold 0.5% bovine-y-globulin (wt/vol in 25 mM Tris-HCl, 1 mM $CaCl_2$; pH 7.4; Sigma Chemical Co.) was added to each tube and vortexed. An equal amount of 20% PEG was then added, tubes were revortexed and centrifuged at 1500 x g for 10 min at 4° C. The supernatants were aspirated and the pellets were resuspended by vortexing in 1.0 ml 25 mM Tris-HCl, pH 7.4, and 1.0 ml PEG was added. The precipitates were collected as described above and the radioactivity present in the pellet was determined in a Packard Gamma Spectrometer.

Characteristics of the specific binding of ^{125}I-hCG to luteal plasma membranes from OA and SA-injected ewes are presented in Figure 3. The curves represent an average of data from OA-injected (panel A) and SA-injected ewes (panel B). These data indicate that a

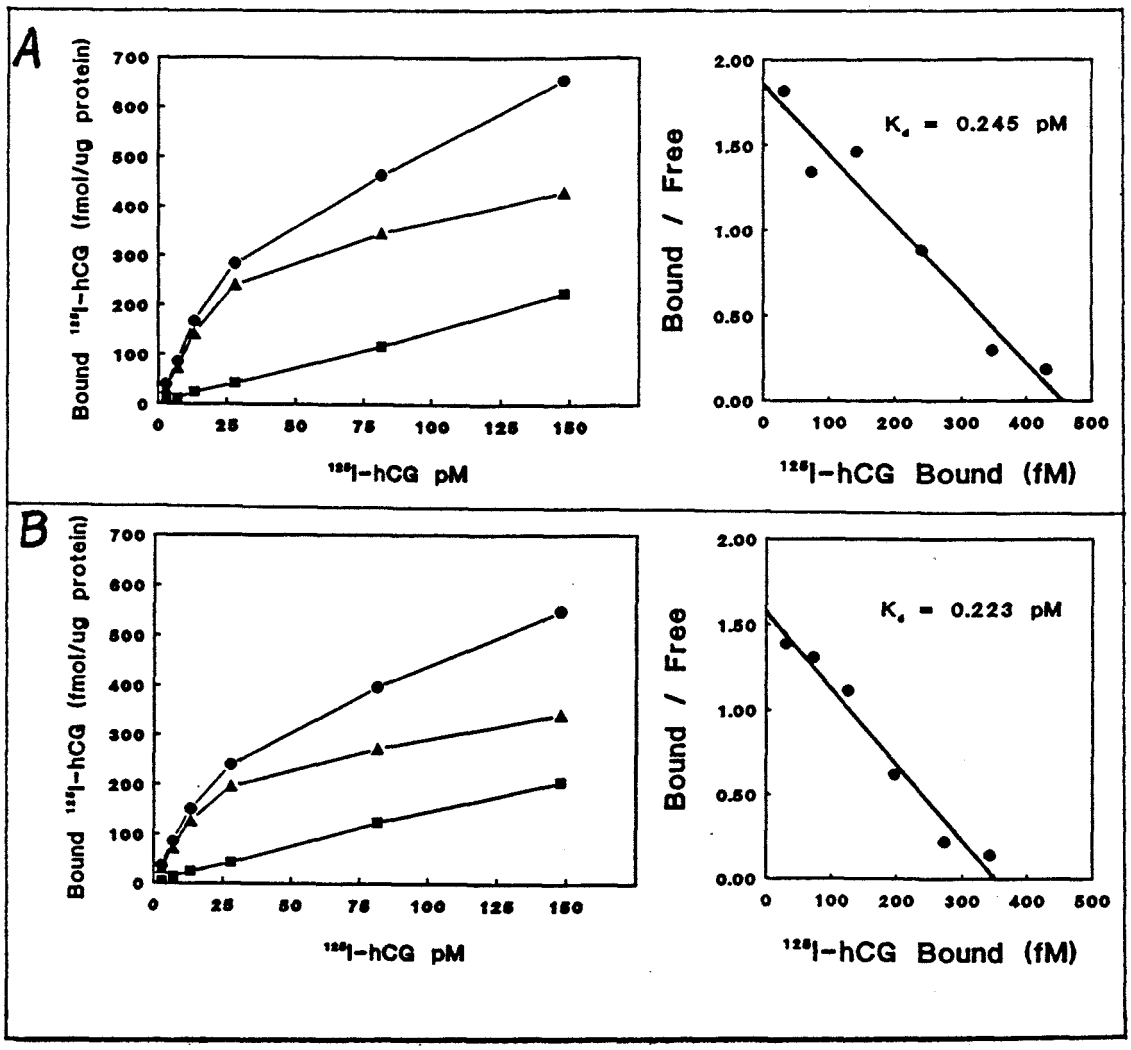

Figure 3. Saturation analyses of ^{125}I-hCG binding to ovine luteal plasma membranes and corresponding Scatchard plots. Increasing concentrations of labeled hCG were incubated in triplicate in the absence (total; —●—●) or presence (nonspecific; —■—■) of a 1000-fold excess of Pregnyl. The difference between total and nonspecific represents specific binding (—▲—▲). Each point is the mean of luteal plasma membranes obtained from oleic acid control (panel A; n = 2) or sterculic acid-treated ewes (panel B; n = 3).

concentration of 54.7 pM ^{125}I-hCG (200,000 cpm) per tube saturated the LH receptors in 50 ug plasma membrane protein.

Scatchard analyses of the saturation data for ^{125}I-hCG specific binding are also depicted in Figure 3. These data reveal linear plots (R^2 = 0.97 and 0.98 for OA and SA-treated ewes, respectively) from which equilibrium dissociation constants (K_d; Figure 3) were calculated. Binding affinities of the receptor for hCG were similar (K_d = 2.45 x 10^{-13} M and 2.23 x 10^{-13} M for OA and SA treatments, respectively) indicating that integrity of the receptor was not affected by SA-treatment.

RADIOIMMUNOASSAYS

Jugular blood samples (10 ml) were allowed to clot at room temperature, stored at 4^{o} C for 24 h and centrifuged at 500 x g for 10 min. Serum was then decanted and stored at -20^{o} C until extracted with hexane:benzene (2:1 v/v) and assayed in duplicate for progesterone (Koligian and Stormshak, 1977). Progesterone (1,2,6,7 [3H]-progesterone, 12 x 10^3 dpm, New England Nuclear: Net-381) were added to a third tube containing an aliquot of each sample to determine procedural loss due to extraction. Extraction efficiency averaged 84.3 + 0.2% with 83 samples. Intra- and

interassay coefficients of variation were 8.5 and 12.0%, respectively.

Extraction of luteal tissue plus medium for progesterone was as follows: To each sample 12 x 10^3 dpm [3H]-progesterone (New England Nuclear, Boston MA) was added to determine the extraction efficiency. The sample (tissue + medium + ethanol) was homogenized in a Duall 24 ground glass homogenizer with an additional 6 ml of absolute ethanol. Samples were filtered through Whatman #1 filter paper with an additional 12 ml of absolute ethanol and then dried under vacuum in a 45° C water bath. The resulting residue was resuspended in phosphate buffered saline with 1% gelatin and allowed to equilibrate for 1 h. Hexane:benzene (2:1 v/v; 20 ml) was added and the sample was vortexed for 2 min then frozen at -20° C for 24 h. The organtic solvent was decanted and dried under air in a 45° C water bath and the remaining residue was redissolved in 20 ml absolute ethanol. A 1 ml fraction of extractant was counted to determine procedural loss. The average extraction efficiency was 86.7% $\pm$ 1.1% in 48 samples.

Progesterone radioimmunoassys were performed on extracted samples following the method of Koligian and Stormshak (1976) utilizing the progesterone antibody (#337 anti-progesterone 11-BSA). Sensitivity of the assay was 10 pg/assay tube (100 ul) and intra- and

interassay coefficients of variation were 4.2 and 5.9, respectively.

STATISTICAL ANALYSIS

Data of Exp. 1 on serum progesterone concentrations were analyzed by split-plot analysis of variance and duration of the estrous cycles was compared by unpaired Students t-test. *In vitro* concentrations of progesterone for Exp. 2 were analyzed by analysis of variance for an experiment of randomized block-split-plot design. Data on unoccupied LH receptor numbers were analyzed by one-way analysis of variance with differences among means tested for significance by unpaired Students t-test.

Results

EXPERIMENT 1

Sterculic acid (750 ug) injected into the ovarian artery of ewes on day 10 of the cycle caused serum concentrations of progesterone to plummet within 12 h (mean $\pm$ SE; control, 2.3 $\pm$ 0.4 vs. treated, 0.3 $\pm$ 0.1 ng/ml) and remain continually suppressed thoughout the remainder of the cycle or until detected estrus ($P < 0.001$; Figure 4). Serum concentrations of progesterone in control ewes were mildly suppressed the day after surgery and then stabilized at below presurgery

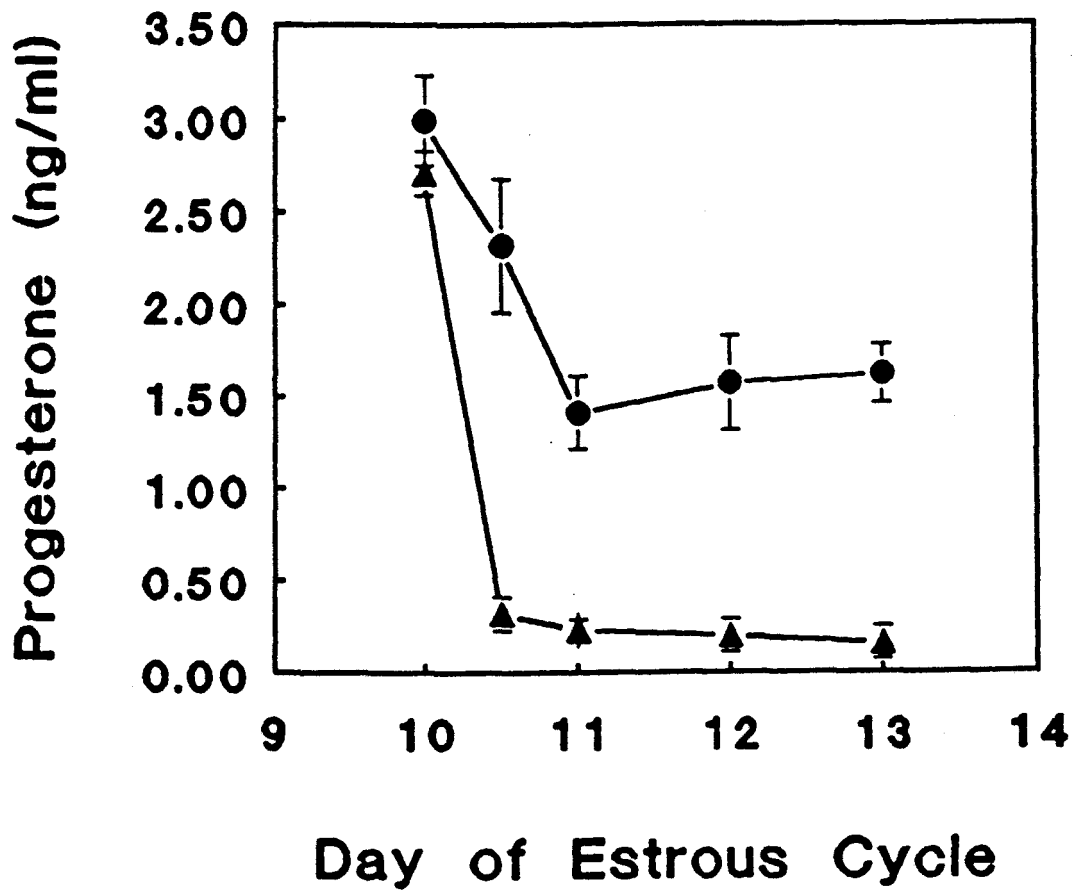

Figure 4. Serum progesterone concentrations (mean + SE) after sterculic or oleic acid injection into ewes on day 10 of the estrous cycle.

concentrations prior to declining during normal luteal regression. Sterculic acid treatment significantly shortened the duration of the estrous cycle (mean ± SE; control, 16.9 ± 0.4 vs. treated, 14.8 ± 0.8 days; $P < 0.05$; Figure 5) but had no effect on duration of the estrous cycle after the treatment cycle (mean ± SE; OA, 16.3 ± 0.3 vs. SA, 16.0 ± 0.4 days).

EXPERIMENT 2

Concentrations of progesterone in luteal tissue collected from ewes on day 10 of the cycle and preincubated with sterculic or oleic acid did not differ after the initial 90 min incubation (unincubated; control 25.2 ± 2.3 vs. treated 25.5 ± 1.6 ng/mg). Progesterone synthesis increased in both SA- and OA-preincubated tissue in response to a subsequent incubation with saline (incubated; control, 37.4 ± 1.8 vs. treated, 37.1 ± 3.3 ng/mg). Relative to progesterone produced during incubation alone, addition of LH or dbcAMP to the incubation medium caused a reduction in steroidogenesis by luteal tissue previously incubated with sterculic acid, but was without effect on progesterone synthesis by luteal tissue preincubated with oleic acid (mean ± SE; ng/mg tissue; OA-oLH, 41.5 ± 3.0 vs. SA-oLH, 27.9 ± 3.1; OA-dbcAMP, 38.0 ± 3.0 vs. SA-dbcAMP, 28.8 ± 2.8; $P < 0.05$; Figure 6). On the average, compared with that of

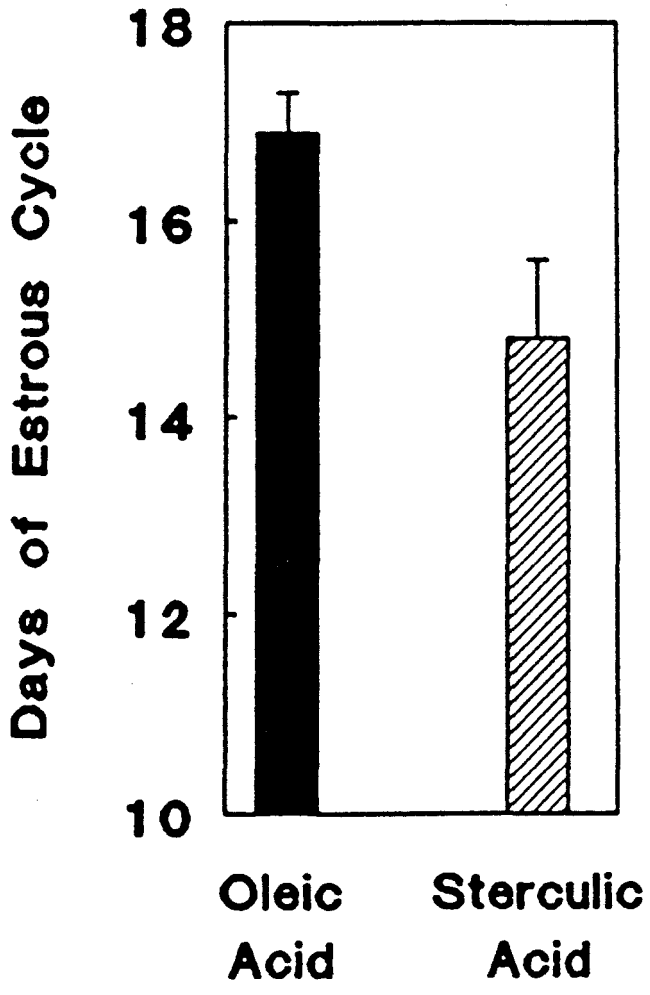

Figure 5. Estrous cycle length (mean + SE) following oleic or sterculic acid administration to ewes on day 10 of the cycle.

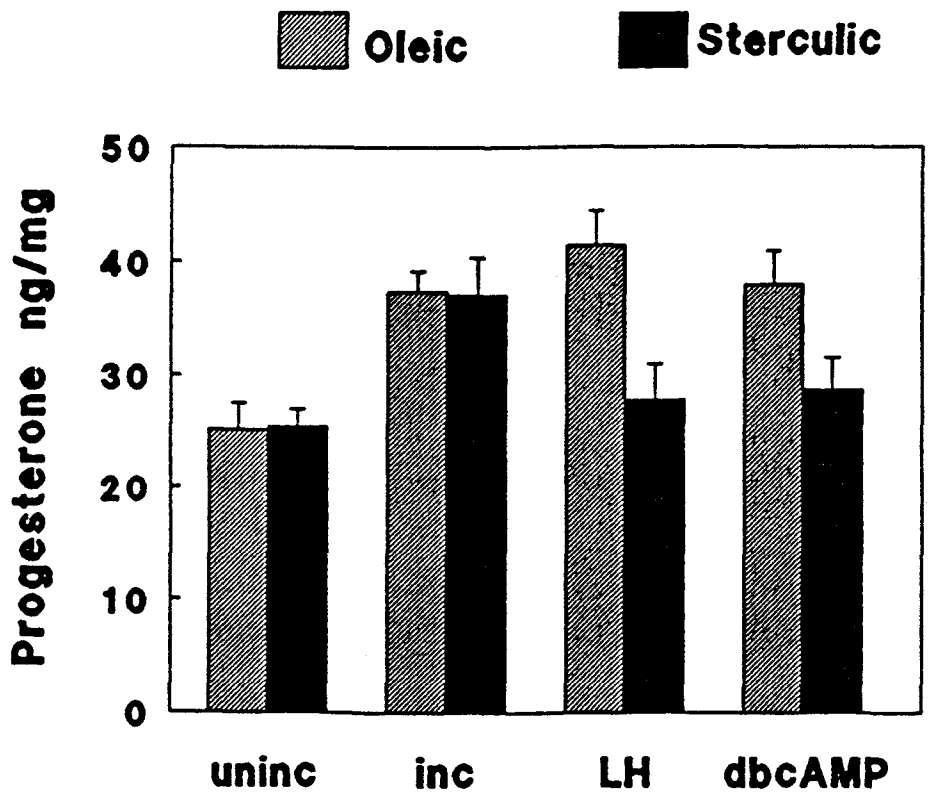

Figure 6. *In vitro* progesterone production (mean + SE) of ovine luteal tissue collected on day 10 of the estrous cycle, preincubated with oleic or sterculic acid and subsequently incubated in the presence of oLH or dbcAMP.

other ewes, luteal tissue collected from one ewe synthesized considerably more progesterone after the second incubation with saline, LH and dbcAMP regardless of whether it was preincubated with SA or OA (OA control, saline 48.6, LH 66.9, dbcAMP 133.4 ng/mg and SA treated, saline 89.5, LH 68.7 and dbcAMP 92.4 ng/mg). The responses of tissue from this ewe to the various *in vitro* treatments were considered to be outliers and therefore data of this animal were not included in the statistical analysis.

EXPERIMENT 3

Concentrations of unoccupied luteal plasma membrane LH receptors for SA-treated ewes were significantly lower than those for OA-injected control ewes (mean ± SE; OA, 161.4 ± 10.8 vs. SA 82.8 ± 16.6 fmol/mg protein; $P < 0.05$; Figure 7). Unoccupied luteal plasma membrane LH receptor concentrations of OA-injected control ewes did not differ from those of corpora lutea on the contralateral ovary of SA-treated ewes (mean ± SE; OA, 161.4 ± 10.8 vs. negative control, 183.0 ± 7.1 fmol/mg protein).

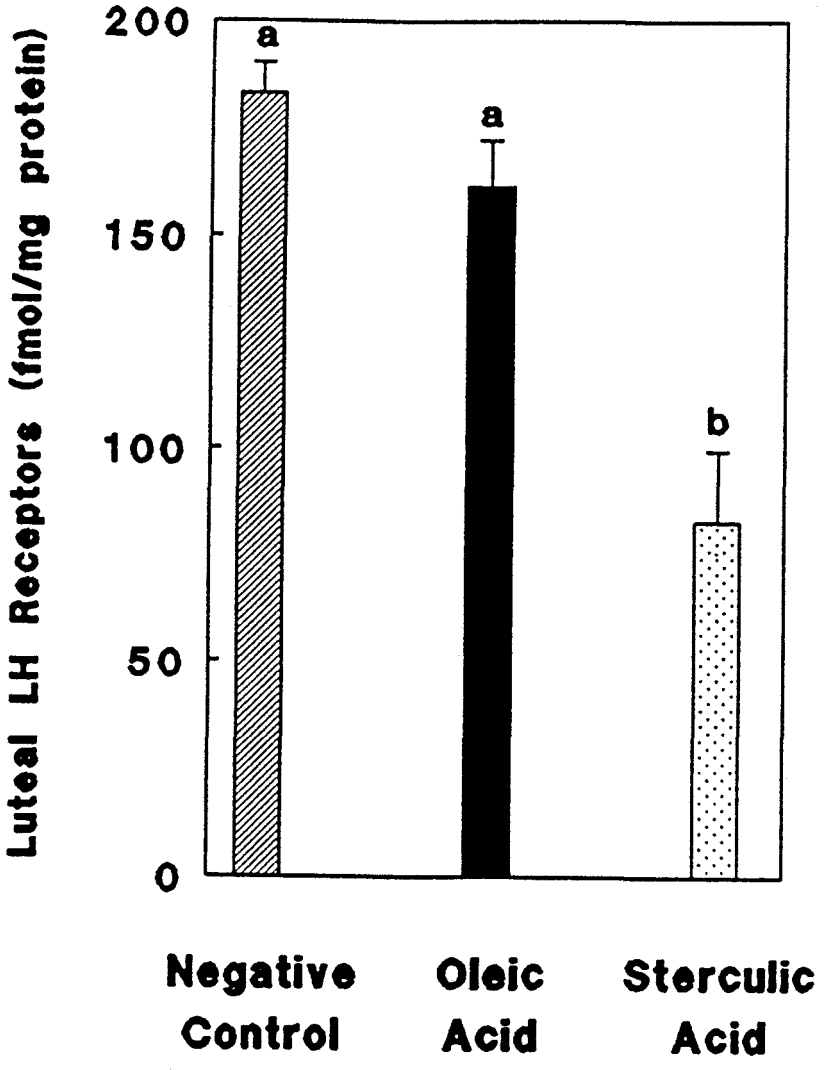

Figure 7. Mean (+ SE) of unoccupied luteal plasma membrane receptor concentrations quantified 30 h after oleic or sterculic acid injection into ewes on day 10 of the estrous cycle.

Discussion

Results of Experiment 1 indicate that *in vivo* exposure of mature corpora lutea to SA interfered with the synthesis and(or) secretion of progesterone, and caused premature luteolysis in a majority of the ewes. The ability of SA to promote premature luteal regression can be attributed to the presence of the propene ring which is absent in the otherwise similar structure of OA. Treatment of ewes with OA, while causing a reduction in serum concentrations of progesterone, did not result in total impairment of luteal function. The initial reduction in progesterone levels after surgery in both OA controls and SA-treated ewes can be explained in part by the removal of CL in the unilaterally ovariectomized ewes. However, because the number of CL removed were essentially the same in each group of ewes, the more dramatic reduction in serum progesterone concentrations in treated ewes must have been due to SA. Estrous cycle duration was shortened in 4 of 6 SA-treated ewes with two returning to estrus within 60 h after SA-injection. None of the control ewes had shortened estrous cycles. Both treated and control ewes had subsequent estrous cycles of normal duration, dismissing the possibility of a carryover effect of treatment.

These data confirm those of Slayden and Stormshak (1988) who found that injection of SA into the ovarian artery of ewes on day 2 of cycle significantly shortened the estrous cycle. Further, a similar injection of SA into ewes on day 18 of gestation attenuated serum concentrations of progesterone and resulted in termination of pregnancy in 3 of 6 ewes (L. Tumbelaka, unpublished results). Collectively, these data indicate that SA can impair the function of the developing and mature corpus luteum and ultimately cause regression of this endocrine gland.

Although the mechanism by which SA inhibits luteal steriodogenesis is basically unknown, the action of this fatty acid does not appear to be the result of altered secretion of luteinizing hormone. Slayden and Stormshak (1988) found that secretion of LH in ewes was not significantly altered by administration of a luteolytic dose of SA on day 2 of the estrous cycle. In further support of this conclusion, preincubation of luteal slices collected from ewes on day 10 of the cycle with SA resulted in a significant suppression of progesterone synthesis when tissue was reincubated with oLH.

The antisteroidogenic effect of SA at the cellular level may involve altered integrity of plasma and mitochondrial membranes. Administration of ^{14}C labeled methyl sterculate to rats resulted in the appearance of

labeled CPFA in significant quantities in the mitochondrial and microsomal fractions of hepatocytes by 4 h after treatment (Nixon et al., 1977). Roehm et al. (1970) had earlier shown that CPFA fed to trout increased the ratio of stearic to oleic and palmitic to palmitoleic in liver triglycerides and phospholipids. These data suggest that the stearic fatty acyl desaturase enzyme system was inhibited by CPFA. Thus, it is not only possible that CPFAs are incorporated into various phospholipids but that they also alter the composition of phospholipids by increasing their concentration of saturated fatty acids. In either case, altered membrane fluidity could prevent normal coupling of the LH-receptor complex to the G_s regulatory protein of adenylate cyclase. This would result in reduced responsiveness of luteal cells to LH as reflected by attenuated progesterone synthesis and secretion.

Although it is possible SA acts proximal to adenylate cyclase to alter membrane fluidity, results from Exp. 2 suggest a site of action distal to adenylate cyclase. This hypothesis is based on the observation that dbcAMP, an analog of cAMP, added *in vitro* after preincubation with SA was unable to enhance progesterone production comparable to levels of controls. If SA was acting only at a site proximal to cAMP, addition of dbcAMP should have replaced the missing link in the

steroidogenic cascade of events resulting in similar progesterone production between treated and control tissues.

Experiment 3 lends further support to a site of SA action distal to adenylate cyclase. Significantly lower concentrations of unoccupied LH receptors after SA treatment compared to OA treatment or the negative controls suggest that SA somehow interfered with recycling or replenishment of the receptors in the plasma membrane. On the other hand, sterculic acid may be altering plasma membrane fluidity and thus preventing endocytocis of the LH-receptor complex. With fewer LH-receptor complexes internalized by the cell, there would be fewer unoccupied LH receptors recycled and available to bind LH. Although results of Exp. 2 did not specifically suggest a site of action of SA proximal to adenylate cyclase, it is still conceivable that SA could cause membrane fluidity changes in addition to acting at a distal site.

A possible alternate and(or) additional site of SA action may be at the smooth endoplasmic reticulum. This was suggested in a recent study by L. Tumbelaka (unpublished results) in which pregnenolone to progesterone conversion was reduced after *in vitro* SA treatment. In addition to its role of converting pregnenolone to progesterone, the endoplasmic reticulum

is also thought to play a role in LH receptor recycling. Not much is known yet about LH receptor recycling and there is presently no evidence indicating that the endoplasmic reticulum is the organelle acted upon by SA to cause the reduction in unoccupied LH receptors. Nevertheless, it is a starting point in designing subsequent experiments to elucidate the organelle(s) and(or) biosynthetic steps affected by SA. Regardless of the mechanism of action, the marked luteolytic effect of this CPFA on the ovine CL may prove to be a useful tool in elucidating the action of endogenous luteolysins.

General Conclusions

Results of Experiment 1 demonstrated that mature corpora lutea are indeed adversely affected by CPFAs, with serum concentrations of progesterone plummeting within 12 h after SA treatment. This rapid reduction in luteal function serves to explain why a majority of these ewes had shortened estrous cycles. In addition to lending support to preliminary CPFA studies conducted in our laboratory, the objectives of Experiments 2 and 3 were to delve for the first time into the mechanism of action of sterculic acid (SA) in the luteal cell. Although these two experiments did not by any means pinpoint the site(s) or mechanism of SA action, they did result in several important findings. Firstly, dbcAMP added *in vitro* after

preincubation with SA was unable to enhance progesterone production to the same extent as found for tissue preincubated with oleic acid (OA), suggesting a site of SA action distal to adenylate cyclase. Secondly, SA treatment caused a reduction in the luteal plasma membrane concentration of unoccupied LH receptors compared to that of appropriate controls, suggesting that SA somehow interfered with recycling or replenishment of the LH receptors. Both of these studies point to a site(s) of SA action within the luteal cell. Obviously, more research needs to be conducted before the specific organelles and the biosynthetic steps affected by this unique fatty acid can be elucidated. However, a starting point to ensure that SA is indeed acting only distal and not proximal to adenylate cyclase would be to measure fluidity of the luteal plasma membrane. Reduced membrane fluidity is a characteristic feature observed during natural or $PGF_2\alpha$-induced luteolysis and it would therefore be interesting to determine whether there is a comparable reduction in membrane fluidity during SA-induced luteal regression.

By elucidating the mechanism of action of SA, it is possible that new insight would be acquired regarding the mechanism(s) by which $PGF_2\alpha$ is able to cause demise of the corpus luteum. Furthermore, by using SA as a tool, it is conceivable that much knowledge could be gained concerning the role of other fatty acids in regulating the life span of the corpus luteum.

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