AN ABSTRACT OF THE THESIS OF

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Abstract approved_____
Fredrick Stormshak

Experiments were conducted to determine whether endogenous progesterone regulates synthesis and/or secretion of luteal oxytocin (OT). In experiment 1, mature ewes (n=5 per group) were assigned randomly to control or mifepristone (RU 486) treatment groups. Ewes were injected twice daily s.c. with vehicle or 10 mg RU 486 from days 5-7 of the estrous cycle (estrus = day 0). On day 8, following an i.v. prostaglandin $F_{2\alpha}$ (250 μg cloprostenol) challenge, venous samples were collected at frequent intervals to determine plasma OT concentrations. Plasma OT in RU 486-treated animals did not differ significantly from those of the control animals ($P > 0.05$). In Experiment 2, ewes were injected s.c. daily with vehicle or 175 mg RU 486 from days 2-5 of the estrous cycle followed by a prostaglandin $F_{2\alpha}$ (250 μg cloprostenol) challenge on day 6. Four of five RU 486-treated ewes exhibited "split-estrus" (estrous behavior

through 36 hours and again 84 to 108 hours after the onset of initial estrus). There was no significant difference in mean plasma OT or progesterone levels between treatment groups ($P > 0.05$). Mean mature corpus luteum (CL) weights of control and RU 486-treated ewes on day 6 did not differ (394.8 ± 28.8 vs. 319.5 ± 48.3 mg; $P > 0.05$). Mifepristone-treated ewes contained mature CL, new CL (2 of 4 ewes), and/or preovulatory follicles ($\geq$ 10 mm, 2 of 4 ewes). Average interestrous interval for RU 486-treated ewes was 9 days longer than that of control animals (26.2 ± 2.9 vs. 17 ± 0.5 days; $P < 0.025$).

A subsequent study was conducted to determine the effects of gonadotropin-releasing hormone (GnRH)-stimulated release of luteinizing hormone (LH) on luteal OT and progesterone production in beef heifers. Ten heifers with normal estrous cycles were assigned randomly in equal numbers to a control and treatment group. On day 2 of the estrous cycle (estrus=day 0) heifers were injected with either physiological saline or 100 μg GnRH every 4 hours for 56 hours. Samples were collected 0 min pre- and 180 min post-GnRH challenge for progesterone analysis. Sixty hours after the initial injection of GnRH or saline, heifers were challenged with an i.v. injection of 500 μg prostaglandin $F_{2\alpha}$ (cloprostenol) and blood was collected at frequent intervals for OT analysis. Luteal OT synthesis was suppressed ($P < 0.01$) in heifers receiving repeated injections of GnRH compared to saline-treated control animals. Progesterone secretion was

significantly greater in saline-treated animals compared to GnRH-treated animals pre- and post-challenge (1.0 $\pm$ 0.06 vs. 0.93 $\pm$ 0.11 ng/ml and 1.16 $\pm$ 0.05 vs. 0.96 $\pm$ 0.13 ng/ml, respectively; $P < 0.05$).

FACTORS AFFECTING LUTEAL OXYTOCIN SYNTHESIS AND/OR SECRETION BY THE OVINE AND BOVINE CORPUS LUTEUM

by
Elizabeth M. Paslay

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There is no place that I can really find to begin. When I embarked upon this journey, after my undergraduate studies, I can honestly say it was because I did not know where I wanted to go in life at that time. A door opened for me when I met a man that suggested I contact Dr. Stormshak and continue my education. Little did I know that these two men would become a very important part in my life, one as a mentor and the other as my friend and husband.

The appropriate place to begin would be with the man who has shaped my mind and career. "Stormy", as we have come to know him, has given me a great opportunity. Through his wisdom, words, and his militant regime in the laboratory and studies, he has built a strong foundation for me to base my future from. The road wasn't always easy, the many questions never seemed to have a straight answer, but he seemed to know when to push my limits and when to guide me when I felt I would break. He and "The Boys", his faithful companions, have given me two years that changed the course of my life for the better. Thanks for your guidance, patience, friendship and most enjoyable disagreements.

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FACTORS AFFECTING LUTEAL OXYTOCIN SYNTHESIS AND/OR SECRETION BY THE OVINE AND BOVINE CORPUS LUTEUM

GENERAL INTRODUCTION

OVERVIEW OF THE OVINE ESTROUS CYCLE

The domestic ruminant estrous cycle consists of four distinct stages that encompass the follicular and luteal phases of the cycle. These four stages are proestrus, estrus, metestrus, and diestrus. The follicular phase, which includes proestrus and estrus, is characterized by declining or low levels of progesterone. Proestrus begins as a result of luteolysis and ends at the onset of estrus. The period of proestrus can last several days, depending on the animal species, and is noted for its hormonal transition from high to low levels of progesterone, and subsequently increasing estrogen levels. Gonadotropins, specifically follicle stimulating hormone (FSH) and luteinizing hormone (LH), are primarily responsible for this hormonal shift. Follicle stimulating hormone and LH recruit follicles to be ovulated, and it is these follicles that secrete large quantities of estrogen that induce the onset of estrus.

Estrus is the most notable phase of the cycle due to behaviors that are displayed during this period. These behaviors occur concomitantly with

the rising levels of estrogen and result in male receptiveness and ovulation. In the ewe, estrus can be detected for 18-48 hours with ovulation occurring 24-30 hours after the onset of estrus (Senger, 1999). Estrus exhibited by the cow lasts about 15-18 hours and ovulation occurs about 10-12 hours after the end of estrus (Senger, 1999).

After ovulation, the follicular cells undergo morphological changes and begin to luteinize after exposure to a surge of LH. The period after ovulation and before the formation of a fully functional corpus luteum (CL) is denoted as metestrus, and is characterized by low levels of estrogen and increasing levels of progesterone.

The CL becomes the dominant "gland" in the ovary during diestrus, secreting massive quantities of progesterone. Progesterone prepares the uterus for embryonic attachment, inhibits ovulation, and male receptivity. This phase is the longest in duration, extending 14-18 days in length depending on the species.

While species like the sow and cow cycle continually throughout the year, some species are affected by season. This seasonal effect on reproduction is especially apparent in the ewe and the mare and can be largely attributed to photoperiod (Hafez, 1952; Kooistra and Ginther, 1975).

These photosensitive species use the number of hours of daylight as a cue to time their reproductive period. The ewe is seasonally polyestrus

and is anestrus during the summer months, beginning to display estrous behavior as hours of daylight become reduced in the fall, and hence, is referred to as a "short-day" breeder. The ewe has a uniform distribution of estrous cycles during the annual period of reproduction beginning in late summer, as the hours of daylight shorten (Hafez, 1952). In contrast, the mare is a so-called "long-day" breeder because estrous behavior begins as hours of daylight increase and ceases during the winter months of anestrus.

OVERVIEW OF OXYTOCIN

Oxytocin (OT) is classified as a peptide hormone, consisting of nine amino acids. This nonapeptide is structurally related to vasopressin (VP) and is believed to be derived from the same ancestral gene, although they are secreted by separate neurons in the hypothalamus. Due to the structural similarities, OT and VP can have the same sites of biological actions due to regional homologies (Norman and Litwack, 1997).

Oxytocin is a product of magnocelluar neurons located in the hypothalamus, and is transported down long axons to be stored in the posterior pituitary until stimulated for release. Ribosomal assembly of oxytocin occurs in the neurons of the paraventricular nuclei, and the

precursor molecules of this hormone are transferred to the Golgi complex to be packaged into neurosecretory granules that move along the axon.

During progression towards the posterior pituitary, post-translational processing of a single gene transcript results in production of VP, OT, and neurophysin (NP) proteins. Oxytocin and VP form a complex with NP; OT complexes with the NP I form, while VP associates with the nearly identical peptide, NP II (Sampson, 2000).

During axonal transport to the posterior pituitary, the signal peptide of the preprotein OT is cleaved by proteases to the NP protein sequence. The prohormone is then further cleaved at the carboxy terminus, resulting in the OT-NP I complex. Neurophysin is believed to stabilize the complex in its granular form during transport to the posterior pituitary for exocytosis, as well as increasing the hormone's half-life in the blood stream (Norman and Litwack, 1997).

Actions of Hypothalamic Oxytocin

Oxytocin has two primary modes of action; milk ejection and uterine contractions during parturition. During the physical stimulus of suckling young, OT is released in response to a rapid spinal arc to the paraventricular nucleus. The stimulatory response is evoked by way of the chemical messenger acetylcholine (Sampson, 2000). However, OT release is inhibited by norepinephrine in response to fear or stress (Sampson, 2000).

During parturition the OT cascade responds to different stimuli. Nearing the termination of gestation, progesterone levels begin to decline drastically. Concomitantly, estrogen levels rise and up-regulate the production of OT receptors (OTR) in the myometrium, increasing the sensitivity of the uterus to OT-induced uterine contractions (Zhang et al., 1992). Due to the increase in OTR, low levels of OT released from the posterior pituitary are effective in stimulating powerful uterine contractions that occur during parturition (Dyer, 1988). However, OT can also stimulate uterine contractions in the nonpregnant animal (Gilbert et al., 1992).

Luteal Oxytocin

The traditional concept is that oxytocin is a neurohormone produced by the hypothalamus, and stored and secreted by the posterior pituitary. However, previous studies have indicated that OT is synthesized in and secreted by the CL of ruminants during the luteal phase of the estrous cycle (Flint et al., 1990).

Wathes and Swann (1982) did not know how correct they were when they hypothesized OT was also an ovarian hormone. Peptides extracted from luteal tissue and assayed for OT indicated that the peptide was indeed OT. In the luteal tissue that was assayed, it was determined that ovarian OT represents about 15% of the total hypothalamo-neurohypophyseal store of hormone in nonpregnant ewes during the luteal phase and roughly 0.2% during pregnancy (Wathes and Swann, 1982). Such a high concentration of hormone within the luteal tissue suggested that it was synthesized there.

Further studies validated this hypothesis when intact and ovariectomized ewes were injected intramuscularly with a synthetic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), cloprostenol, and ovarian venous blood samples were collected. Within 10 minutes of administration of $PGF_{2\alpha}$ to intact ewes, there was a transient rise in plasma OT that lasted up to 40 min (Flint and Sheldrick, 1982). However, this transient increase in OT was absent in

ovariectomized ewes. Administration of $PGF_{2\alpha}$ was unable to stimulate secretion of OT by the posterior pituitary in the ovariectomized ewes, confirming that the CL was the source of the peptide hormone during the luteal phase of the estrous cycle (Flint and Sheldrick, 1982; Schams et al., 1982).

LUTEAL OXYTOCIN SYNTHESIS

In the hypothalamic-neurohypophyseal system, OT is synthesized as a precursor molecule along with NP I (Richter, 1983). Ovarian OT collected from bovine luteal extracts and utero-ovarian plasma has been shown to contain immunoreactive NP I (Wathes et al., 1983; Swann et al., 1984). Previous research has determined that luteal OT, and its associated NP, is released in a 1:1 equimolar equivalence upon exocytosis, just as in the posterior pituitary (Schams et al., 1985b). This simultaneous release indicates a potentially similar synthesis mechanism in the CL as in the brain. Neurophysin I and OT are always secreted concomitantly in major quantities from the CL and in minor quantities from follicles; the concentration depending on the number of CL or follicles present, not on the rate of progesterone secretion (Schams et al., 1985a).

Oxytocin and its associated NP have been localized in secretory granules of large luteal cells (Fields and Fields, 1986). When granulosa cells, theca cells, and endothelial cells were analyzed by electron microscopy, secretory granules of OT-NP were identified in the granulosa cells only (Theodosis et al., 1986). Immunocytochemistry failed to detect OT in tissues such as the corpus albicans or liver cells (Jones and Flint, 1988). Luteal OT granules are larger in size than those of the posterior pituitary, containing a two-part core structure to distinguish them from the neurosecretory granules (Theodosis et al., 1986).

Oxytocin and its associated NP have been localized in secretory granules of granulosa cells (Wathes and Swann, 1982; Swann et al., 1984; Theodosis et al., 1986; Fields and Fields, 1986). These granules are formed in the Golgi apparatus and migrate to the membrane for exocytosis, consistent with secretion of other peptide hormones from endocrine tissues (Fields and Fields, 1986). It has been suggested that NP may serve to stabilize OT molecules, preventing intragranular degradation as suggested for OT granules of the posterior pituitary (Shuckovski et al., 1991). Little is known about the specific biological actions of the luteal NP. Shuckovski et al. (1991) also found evidence that biologically active luteal OT is associated, in non-covalent fashion, with NP I in the luteinizing granulosa cells of the cow. This non-covalent binding occurs in the granule, and non-

enzymatic separation of OT from NP I represents the last phase of post-translational processing (Sheldrick and Flint,1989; Shuckovski et al., 1991).

LUTEAL OXYTOCIN GENE EXPRESSION

Expression of OT genes had been described thus far only in the hypothalamus, however, it was not clear whether or not the OT found in the CL was taken up from circulation to be released later on specific stimulation or synthesized within the tissue. Using a combination of DNA hybridization, cDNA cloning, and *in vitro* translation, OT mRNA was identified in the bovine and ovine CL, with the greatest amount occurring around day 3, after estrus (Ivell and Richter, 1984; Jones and Flint, 1988). From cloned DNA, Ivell and Richter (1984) were able to determine that luteal OT mRNA is similar to that of hypothalamic origin and is not a product of a second or alternative gene. This experiment also confirmed that the OT gene is actively transcribed in the bovine and ovine CL (Jones and Flint, 1988).

The process of luteinization involves exponential growth of cells and is accompanied by the differentiation of the follicle wall. Corner (1919) observed that the CL contained the same cells as the follicle. However, early after the rupture of the follicle Corner (1919) also observed that the

membrana propria that separates the thecal and granulosa cells breaks down. Theca interna cells mulitiply in number and disperse between the granulosa cells that are undergoing hypertrophy (Corner, 1919). Theca and granulosa cells migrate inward to form the CL, which steadily increases in size following ovulation.

Although granulosa cells *in vivo* appear to contain a low level of OT mRNA, gene transcription is upregulated on the day of ovulation to yield 100-fold higher levels of OT mRNA by day 3 of the bovine cycle (Furuya et al., 1990). Bovine granulosa cells isolated after the LH surge secrete much greater quantities of OT than those isolated before the surge (Voss and Fortune, 1991). In addition, OT mRNA was found to be significantly increased in cells collected after the preovulatory surge of LH (Voss and Fortune, 1992). However, there is no correlation that can be found between the level of OT mRNA and that of the peptide within the CL (Fehr et al., 1987). Experimental evidence suggests that there is a delay in the peak of OT peptide production 6-7 days after the OT mRNA peak, which coincides with maximal synthesis of luteal progesterone (Fehr et al., 1987).

Oxytocin and its associated NP gene expression occur at a high rate at or shortly after luteinization, with transcription rates markedly reduced thereafter (Jones and Flint, 1988). Further experiments reaffirmed the presence and active transcription of the OT gene. When hypothalami,

corpora lutea, corpora albicans, and follicles from all stages of the bovine estrous cycle were extracted for RNA, autoradiograms revealed the expression of the gene in low amounts in mid-cycle follicles (Ivell et al., 1985). Furthermore, Ivell and associates (1985) supplied further evidence supporting the observation that ovulation causes a surge of total RNA production concomitant with the growth of the luteinizing tissue peaking at day 3, and declining thereafter by day 11.

Due to the up-regulation in OT mRNA directly after ovulation, researchers began to investigate the possibility that the hormones involved in luteinization may also affect the transcription of the OT gene. Insulin-like growth factor-I (IGF-I) and insulin increased levels of OT mRNA and stimulated the release of OT and progesterone *in vitro* (Holtorf et al., 1989; Furuya et al., 1990; Fleet et al., 1994). These results could only be obtained from cells collected from highly differentiated granulosa cells; i.e., cells from a follicle containing at least 40 ng/ml of estrogen in follicular fluid, which up-regulates LH receptors (Holtorf et al., 1989; Furuya et al., 1990; Voss and Fortune, 1991). In contrast, there was no effect of IGF-1 or insulin in less mature granulosa cells of follicles containing less than 1 ng/ml of estrogen (Holtorf et al., 1989).

When highly differentiated granulosa cells cultured *in vitro* were exposed to IGF-I, OT mRNA increased and remained elevated for an

extended period of time (Furuya et al., 1990). However, when these researchers exposed granulosa cells to estrogen and $PGF_{2\alpha}$, these hormones had no effect on OT mRNA, irrespective of whether or not they were pretreated with IGF-I. In the absence of IGF-I, OT release and specific mRNA levels declined in a shorter amount of time. Furuya and associates (1990) concluded IGF-I was a necessary component in the up-regulation of gene transcription for luteal OT and that estrogen and $PGF_{2\alpha}$ had no effect on the transcription of the luteal OT gene. Attempts to induce mid-phase luteal cells to synthesize OT failed to yield any OT mRNA, though peptide release could be measured, suggesting that luteal OT may be released from a prohormone store (Furuya et al., 1990; Voss and Fortune, 1992).

Further studies revealed the importance of follicular stage of development on the secretion of OT. Granulosa cells isolated before the preovulatory surge of LH responded with a marked increase in OT secretion when stimulated by FSH and LH *in vitro* (Voss and Fortune, 1991). Their research also demonstrated that OT was not detectable in theca interna cells isolated before or after the LH surge (Voss and Fortune, 1991; 1992). These data led researchers to conclude that OT-NP I mRNA is not accumulated in the CL during the mid-luteal phase of the cycle, but rather earlier in the luteal phase with a delay in translation and OT synthesis.

PROGESTERONE, PROGESTERONE ANTAGONISTS, AND PROGESTERONE INHIBITORS

Hormone antagonists and inhibitors are useful research tools. The synthetic steroid mifepristone (RU 486) is a progesterone antagonist that has been proven to be very effective, binding to progesterone receptor (PR) sites and blocking the action of endogenous progestins in humans and primates (Healy et al., 1983; Shoupe et al., 1985) as well as sheep (Burgess et al., 1992; Morgan et al., 1993) and rats (Fang et al., 1997). Mifepristone has been shown to bind to PR in the uterus of pregnant and nonpregnant ewes with very high affinity, inhibiting the actions of progesterone (Burgess et al., 1992; Morgan et al., 1993). Many studies have utilized RU 486 to investigate the effects of progesterone withdrawal during various times of the estrous cycle and gestation.

Ewes receiving RU 486 during late gestation experienced induced labor and parturition following an OT challenge compared to control animals (Burgess et al., 1992). Plasma samples of ewes treated with RU 486 revealed enhanced levels of 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM), a $PGF_{2\alpha}$ metabolite, in response to the OT challenge while progesterone levels remained the same between both treatment groups. Burgess et al. (1992) hypothesized that by blocking the effect of progesterone at the level

of its receptor, the ability of endogenous estrogen to enhance the oxytocin receptor (OTR) would not be inhibited by endogenous progesterone.

Morgan et al. (1993) conducted a study to test the hypothesis that exposure to progesterone in early to mid-diestrus regulates uterine release of $PGF_{2\alpha}$ at the time of luteolysis in sheep. They determined that pulsatile secretion of PGFM was inhibited in those animals receiving RU 486. This inhibition of $PGF_{2\alpha}$ resulted in a failure of luteolysis and prolonged the duration of the cycle until day 24 after estrus (Morgan et al., 1993). This study demonstrated that RU 486 inhibits the function of uterine PR, delaying the down-regulation of the PR, therefore postponing luteolysis.

Two potent progesterone inhibitors, trilostane and epostane, have also been utilized to study the effects of progesterone during the estrous cycle and gestation of ewes (Hoefler et al., 1986; Webb, 1987) and cattle (Peters and Lamming, 1986), and during the menstrual cycle of primates (Schane et al., 1979). Trilostane and epostane are inhibitors of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), a crucial enzyme in the synthesis of progestins, estrogens, androgens, and glucocorticoids. These enzyme-inhibiting compounds block the activity of the enzyme by competing with pregnenolone for binding sites on the 3β-HSD molecule (Peters and Lamming, 1986).

Use of trilostane in ewes has been effective in decreasing serum progesterone levels; however, epostane has been shown to be the most effective. While trilostane decreases progesterone levels from approximately 10 to 3 ng/ml, its effects only last about 4 to 6 hours after injection (Jenkin et al., 1983). Epostane decreased serum progesterone levels to about 1 ng/ml for up to 65 hours post-injection (Ashworth et al., 1986). Although both drugs caused an inhibition in serum progesterone levels, various doses of trilostane or epostane failed to induce structural luteolysis (Jenkin et al., 1983; Ashworth et al., 1986; Hoefler et al., 1986; Peters and Lamming, 1986)

Recently there has been increasing evidence delineating the exact role progesterone plays in regulating luteal OT synthesis. Smith et al. (1995) have identified the presence of progesterone receptors (PR) within granulosa cells of ovine follicles and CL. However, PR mRNA was nondetectable in follicles collected before the LH surge, suggesting a role of progesterone during the preovulatory period and luteal phase in sheep.

In reviews published by Walther et al. (1995) and Gimpl and Fahrenholz (2001), these investigators reported the finding of a hexanucleotide sequence response element within the promoter region of the OT gene. Variations of this six nucleotide sequence among species, represent part of a binding site for the steroid nuclear receptor superfamily.

However, glucocorticoid, mineralcorticoid, progesterone and androgen receptors do not interact with the OT gene at this sequence and do not have any known direct effects on the transcription of the OT gene at this time (Gimpl and Fahrenholz, 2001). It has been reported that many orphan receptors in the steroid nuclear receptor superfamily may interact with the OT gene, affecting the regulation of gene expression (Gimpl and Fahrenholz, 2001). A highly conserved sequence, the estrogen response element (ERE), could be activated by the binding of estrogen in the rat and human (Gimpl and Fahrenholz, 2001). In similar ligand-activated gene regulation studies conducted in the bovine, the ERE promoter sequence was not present and there was no direct effect by ER activation (Gimpl and Fahrenholz, 2001). It remains unclear if members of the steroid nuclear receptor superfamily exert direct transcriptional effects on the bovine OT gene.

Although there is evidence of PR mRNA present in membrane fractions of granulosa cells from ovine follicles and corpora lutea, researchers consistently failed to demonstrate binding of progesterone, progesterone agonists, or progesterone antagonists to the steroid receptor in granulosa membrane fractions (Rae et al., 1998a). However, binding of progesterone did occur in this study when the membrane fractions were incubated in the presence of digitonin.

The presence of PR in the follicular and luteal cells of the ovary (Smith et al., 1995; Lioutas et al., 1997) suggests that progesterone may play an autocrine role in regulating OT gene upregulation. The research of Lioutas et al. (1997) demonstrated that addition of progesterone to cultured bovine granulosa cells increased cumulative OT secretion, suggesting that progesterone is acting in an autocrine fashion to mediate OT up-regulation.

In a similar study, bovine granulosa and theca cell membranes from follicles at different stages of the estrous cycle were isolated and specific binding to the membrane fractions were assessed. Again, poor binding of progesterone occurred unless cells were incubated in the presence of digitonin (Rae et al., 1998b). Rae et al. (1998b) also examined the binding of cholesterol, and the progesterone antagonist RU 486, and determined that they too competed poorly for the binding site. These researchers determined that binding of progesterone to granulosa cell membranes decreased significantly with increasing follicle size and suggested that these membrane steroid receptors may be involved in regulation of follicular function.

It has been determined through cloning of the luteal PR that high homology exists among bovine, ovine, human, mouse, rabbit and rat nuclear receptors (Bolden-Tiller et al., 2000). The presence of PR in the nuclei of cells was confirmed by use of immunocytochemistry and ligand

binding assays. Additional evidence confirming the intracellular location of the PR was obtained when the progesterone antagonist RU 486 competed for binding to this receptor within the uterus of the ewe (Rauch et al., 1985; Bailly et al., 1986).

Bovine granulosa cells were cultured and examined to determine whether or not progesterone may be acting in an autocrine fashion on luteal cells. When stimulated with LH, the cell cultures proceeded to produce large amounts of OT and progesterone (Lioutas et al., 1997). Progesterone antagonists, RU 486 and onapristone, when added to cell cultures inhibited progesterone binding; but these antagonists had no effect on the levels of estrogen or progesterone produced by the cells (Lioutas et al., 1997). Mifepristone significantly reduced OT secretion in luteal cell cultures and this effect could only be reversed in the presence of a synthetic progesterone; the addition of dexamethasone had no effect. These researchers analyzed OT mRNA and determined that RU 486 was inhibitory at the level of gene transcription. Lioutas et al. (1997) concluded that progesterone is acting in an autocrine fashion to promote OT up-regulation. In summary, OT and progesterone are hypothesized to be components of a luteal autocrine positive feedback loop, beginning with LH to stimulate steroidogenesis and resulting in progesterone acting to regulate OT gene expression.

LUTEAL OXYTOCIN SECRETION

Luteal OT is temporarily stored in the bovine and ovine corpora lutea in granular form during the luteal phase of the cycle. However, within 10 minutes of administering $PGF_{2\alpha}$ to the ewe or cow, there is an increase in OT secretion (Flint and Sheldrick, 1982; Wathes et al., 1983). It has been demonstrated that luteal OT secretion is stimulated by $PGF_{2\alpha}$ and may be critical for luteal regression in the ewe (Flint and Sheldrick, 1990).

Various hormones mediate initiation of luteolysis. Just prior to estrus in the ewe, high levels of estrogen from the growing follicles stimulate the up-regulation of OTR in the uterus (Roberts et al., 1976; Sheldrick and Flint, 1985; Vallet et al., 1990; Zhang et al., 1992). After ovulation and during the formation of the corpus luteum, LH causes a shift from estrogen production to progesterone production in the luteinizing cells, and a significant increase in luteal OT (Voss and Fortune, 1991, 1992). This increasing progesterone production down-regulates OTR in the uterus (Sheldrick and Flint, 1985; Zhang et al., 1992) and has been shown to stimulate OT secretion from granulosa cells *in vitro* (Lioutas *et al.*, 1997; Al-Matubsi et al., 1998).

It has been determined in the ewe that prior exposure to increased systemic levels of progesterone was essential for the endometrium to respond with an up-regulation of uterine OTR when exposed to estrogen

(McCracken, 1980; Vallet et al., 1990; Zhang et al., 1992). The priming effects of progesterone were apparent when ovariectomized ewes were injected with a sequential combination of progesterone and estrogen or estrogen alone (Zhang et al., 1992). The results indicated that there was a significant increase in the concentration of endometrial OTR present in the ewes that were previously exposed to progesterone compared to ewes receiving estrogen alone (Zhang et al., 1992). This research validated the hypothesis that the normal response and changes in OTR concentrations of the endometrium to progesterone and estrogen requires prior exposure to progesterone (Vallet et al., 1990).

In ovarian autotransplanted ewes, administration of progesterone on days 1-3 of the luteal phase resulted in significant increases in the pulses of OT and $PGF_{2\alpha}$, stimulating early luteolysis as compared to control animals (Al-Matubsi et al., 1998). These researchers also determined that the uterus must be exposed to increased levels of progesterone for 7-8 days before the uterus will respond to OT with a release of $PGF_{2\alpha}$. Intact ewes receiving progesterone early in the luteal phase also exhibited an increase in the number of spontaneous OT surges suggesting that progesterone may play a role in regulating the timing and release of ovarian OT (Fairclough et al., 1983; Al-Matubsi et al., 1998).

Secretion of luteal OT comes from the large luteinized granulosa cells in the cow and ewe (Swann et al., 1984; Theodosis et al., 1986; Fields and Fields, 1986; Shuckovski et al., 1991). Granulosa cells isolated from bovine preovulatory follicles were cultured and exposed to OT to determine whether or not luteal OT plays a role in regulating steroid synthesis. Oxytocin markedly inhibited estrogen (Chandrasekher and Fortune, 1990) and stimulated progesterone synthesis (Berndston et al., 1996); however, mRNA for P 450 side chain cleavage enzyme, P 450 aromatase enzyme, and 3β-HSD were not affected (Berndtson et al., 1996). When estrogen was studied to determine its effects on OT secretion, it was determined that the results were dependent on the stage of the cycle. Estrogen stimulated OT secretion in ovine preovulatory follicles *in vitro* and inhibited OT synthesis in day 1 CL and during the early luteal phase CL (Wathes et al., 1992). Capacity for OT secretion is confined to healthy follicles and is lost during atresia (Jungclas and Luck, 1986).

There is a high concentration of OT in the bovine CL during the estrous cycle, with the highest concentration being present during the midluteal phase (Schams et al., 1985; Abdelgadir et al., 1987). Although high progesterone levels accompany the midluteal phase increase in luteal OT, Jungclas and Luck (1986) concluded that OT production does not always accompany increased progesterone secretion. However,

Chandrasekher and Fortune (1990) determined that cultured bovine granulosa cells treated with an OT antagonist responded with significantly lower progesterone levels than cells treated with OT alone or in combination with the antagonist. These researchers concluded that OT may somehow facilitate the shift in steroidogenesis from estrogen production to progesterone production.

Prostaglandin $F_{2\alpha}$ stimulates the release of luteal OT from bovine (Schallenberger et al., 1984; Orwig et al., 1994a; Salli et al., 2000) and ovine (Flint and Sheldrick, 1982; Wathes et al., 1983) CL. Therefore, synthetic agents mimicking the effects of $PGF_{2\alpha}$ should elicit a release of OT via intracellular signals. In a study conducted by Cosola-Smith et al. (1990), the effects of phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which has structural similarities to DAG and is able to directly activate protein kinase C (PKC), and a calcium ionophore (A23187), were utilized to study the secretion of OT from bovine luteal tissue *in vitro*. Results indicated that TPA and the calcium ionophore could stimulate an *in vitro* secretion of OT. These studies suggested a role of PKC in luteal OT secretion, as well as the importance of intracellular calcium for the enhancement of the secretion of OT from bovine luteal cells.

Studies have also been conducted to examine the specific mechanisms involved in the exocytosis of OT granules from the bovine CL.

Previous studies were conducted to determine which PKC isoforms are present and their distribution within the bovine CL (Orwig et al., 1994b). Orwig et al. (1994b) determined by western blot analysis that the bovine CL expressed conventional PKC α and novel PKC ε isozymes. Although the roles of these specific PKC isozymes were not elucidated, it was proposed that they played a role in the exocytosis of OT.

Salli et al. (2000) studied the disassembly of the actin cortex of the luteal plasma membrane to determine specific mechanisms involved in the exocytotic process. These researchers identified a role for the myristoylated alanine-rich C kinase substrate (MARCKS) protein that crosslinks the actin cortex filaments (Hartwig et al., 1992). When phosphorylated by PKC, MARCKS translocates from the plasma membrane to the cytoplasm causing a disassembly of the actin cortex and thus facilitating OT secretion. These events have been observed within bovine luteal cells stimulated by $PGF_{2\alpha}$ during the midluteal phase of the estrous cycle, suggesting that exposure to $PGF_{2\alpha}$ must result in activation of PKC.

Various studies have been conducted to examine the effect of different peptide hormones on the synthesis and secretion of OT. Activin-A, an ovarian peptide, has been shown to cause a time-dependent decrease in progesterone and OT secretion with no significant difference among doses utilized *in vitro* (Shuckovski and Findlay, 1990). These latter

investigators made the novel observation that activin-A may act as a luteinizing inhibitor *in vivo*, although the biological implications have not been assessed.

Miyamoto et al. (1993) examined the effects of various neuropeptides in *in vitro* cultures of midluteal bovine granulosa cells. They found that LH alone or in combination with neuropeptide Y, substance P, or vasoactive intestinal polypeptide, had no effect on OT secretion in cell culture in the long or short term. They also concluded that these neuropeptides were stimulatory for progesterone production but not OT synthesis.

Due to the presence of adrenergic sympathetic nerves within the ovary, in particular the ruminant ovary (Stefenson et al., 1981), investigators began to assess the effects of various biogenic amines on luteal OT. Bovine granulosa cells were cultured and OT production was measured by secretion into the medium. Adrenaline and noradrenaline stimulated OT secretion and progesterone was significantly reduced, while acetylcholine had no effect on OT production (Luck and Jungclas, 1987; Heap et al., 1989). Ascorbate, was also examined at the same time and it was determined that alone, it produced a smaller increase than that of the biogenic amines themselves (Luck and Jungclas, 1987). However, Heap et al. (1989) found that although adrenaline and noradrenaline stimulated OT

production in sheep, they were also able to increase OT production with acetylcholine. These researchers concluded that neurotransmitter-induced OT release *in vivo* is rapid, episodic, and closely associated with reduced blood flow arising from acetylcholine stimulation.

UTERINE OXYTOCIN RECEPTOR

The OTR is classified as a seven-membrane-spanning receptor, or a G-protein coupled receptor. The OTR G-protein is coupled to the phospholipase C (PLC) system and forms part of the subfamily of neurohypophyseal hormone receptors that includes vasopressin receptors (VPR). These OTR are located in the uterus, pituitary, and the hypothalamus, as well as mammary tissues (Zingg et al., 1998).

As the receptor site becomes occupied, the receptor changes conformation, interacting with a G_q-protein. The G_q-protein consists of three proteins designated α, β, and γ. Activation of the OTR stimulates the dissociation of $G_{\alpha q}$, which in turn activates PLC-β. Phospholipase C-β activates the phosphoinositide cascade, generating inositol trisphosphate and 1,2-diacylglycerol (DAG). Diacylglycerol is located in the membrane, causing activation of phospholipase A_2, and in the case of the uterine OTR,

generates production and release of uterine $PGF_{2\alpha}$ from arachidonic acid precursors (Watanabe et al., 1985). Activation of inositol trisphosphate, triggers the release of intracellular calcium to activate PKC in conjunction with DAG (Flint et al., 1986; Gimpl and Fahrenholz, 2001). Protein kinase C can then phosphorylate other target proteins.

To further elucidate the regulatory mechanisms of the uterine OTR, studies conducted by Hazzard and Stormshak (1997) suggested that chronic exposure of ewes to estrogen during the estrous cycle down-regulates the concentration of uterine OTR but not estrogen receptors (ER) and reduces the OT-induced release of $PGF_{2\alpha}$, thus prolonging the life span of the CL. These data were confirmed in part by studies revealing that chronic treatment of ewes with estrogen from early to late in the estrous cycle prevented a normal increase in OTR gene transcription (Hazzard et al., 1998). The suppressive effects of chronic estrogen exposure on the uterine OTR are hypothesized to be a distinctly different mechanism than that observed during progesterone exposure during the late luteal phase of the ovine estrous cycle. However, there is no evidence to date of any inhibitory effects of chronic estrogen on the secretion of OT or the ability of the CL to secrete OT.

Studies have demonstrated that regulation of uterine OT binding involves estrogen-induced up-regulation and progesterone-induced down-

regulation (Zingg et al., 1998) of OTR. Currently, all that is known about luteal OT is its stimulatory effect on uterine $PGF_{2\alpha}$ and the luteolytic effect of the latter hormone on the CL. As of now, scant knowledge exists about the intracellular changes that lead to cell death during the induction of luteolysis in the cow and the ewe.

EFFECTS OF OXYTOCIN ON THE UTERUS

Over the past three decades numerous and extensive studies have determined that the uterus exerts its effects on the ovary, more specifically the CL, by a local action (Brinkley et al., 1964; Collins et al., 1966; Inskeep et al., 1966). An example of this local action was presented by Inskeep and Butcher (1966) with ewes that were unilaterally hysterectomized ipsilateral to the CL, or unilaterally ovariectomized. Unilateral ovariectomy had no effect on the length of the estrous cycle. However, ewes that were unilaterally hysterectomized alone or in combination with a contralateral ovariectomy, displayed a prolonged life span of the CL. At the time, these researchers hypothesized that there may be a substance that travels between the uterus and ovary via the blood, that regulates luteal life span.

This substance would later be identified as the luteolytic factor $PGF_{2\alpha}$ (McCracken et al., 1972; Schallenberger et al., 1984).

A study was conducted to further elucidate the relationship between the ovary and uterus. To determine whether or not precocious estrous could be induced via the utero-ovarian relationship in cattle, OT was administered (Ginther et al., 1967). These researchers hypothesized that OT would cause precocious estrus in unilaterally hysterectomized heifers if the retained horn was adjacent to the ovary with the CL. These researchers concluded that OT administered to intact or unilaterally hysterectomized animals would indeed shorten the estrous cycle. This further implicated the role of luteal OT and the local actions of uterine $PGF_{2\alpha}$.

Prostaglandin $F_{2\alpha}$ has been identified as the luteolytic hormone in sheep (McCracken et al., 1972) and cattle (Schallenberger et al., 1984). Luteal OT, in concert with uterine $PGF_{2\alpha}$, are two major factors involved during the regression of the CL of ruminants, at least in the ewe (Roberts *et al.*, 1976; Roberts and McCracken 1976; McCracken *et al.*, 1996). Although it is not exactly known what causes the initial release of uterine $PGF_{2\alpha}$, the effecter has been proposed to be a surge release of OT from the posterior pituitary (McCracken et al., 1996). Luteal OT, released in response to the first surge of uterine $PGF_{2\alpha}$, targets the uterus stimulating

another surge release of $PGF_{2\alpha}$; sequential releases of $PGF_{2\alpha}$ eventually result in luteal regression (McCracken et al., 1972; Roberts et al., 1976). The manner in which these two hormones work together that results in luteal regression has been termed a "double-positive feedback loop" (McCracken et al., 1972).

EFFECT OF PITUITARY HORMONES ON LUTEAL OXYTOCIN

Gonadotropin releasing hormone (GnRH), also known as luteinizing hormone releasing hormone (LHRH) / follicle stimulating hormone releasing hormone (FSHRH), is a decapeptide hormone located within cell bodies of the medial basal hypothalamus and the preoptic areas. However, in primates GnRH is also localized within cell bodies of the arcuate nucleus (Griffin and Ojeda, 2000).

Axons from the cell bodies of the hypothalamus carry neuropeptides to a capillary bed at the base of the pituitary stalk. This system is known as the hypothalamo-hypophyseal portal system (Senger, 1999). This complex network of capillaries carry minute quantities of releasing hormones to the anterior pituitary where they stimulate cells to secrete a variety of hormones.

These various pituitary hormones are released directly into the blood stream from the anterior pituitary. The principal reproductive hormones released from the anterior pituitary in response to GnRH are FSH and LH. These anterior pituitary hormones target the gonads to regulate responses in the female such as follicular growth, ovulation and luteal development.

Rahe et al (1980) evaluated endogenous patterns of LH secretion in the cow during different stages of the estrous cycle. These researchers concluded that serum LH fluctuated in a pulsatile manner throughout all periods of the cycle. Early in the estrous cycle, LH was observed to pulse in low amplitude but high frequency with a mean serum level between 1.3 and 2.2 ng/ml (Rahe et al., 1980). However, as the cycle progressed, midluteal phase pulses were observed to be of high amplitude and low frequency; and during the late luteal phase these pulses were characterized as high amplitude, high frequency pulses resulting in the ovulatory surge.

Many studies have utilized GnRH in an attempt to induce estrus in anestrous animals. Others have evaluated the effect of GnRH on luteal function through the effects of LH (Adams et al., 1975; Kinder et al., 1975; Rodger and Stormshak, 1986; Slayden and Stormshak, 1990). Constant GnRH infusion into ewes over an extended period of time was not sufficient to maintain serum LH at concentrations sufficient to stimulate ovulation (Chakraborty et al., 1974). When these ewes were pretreated with

progestagens, there was still no effect on the stimulation of ovulation. Chakraborty et al. (1974) concluded that pulsatile injections of GnRH, mimicking the pulsatile-type release from the hypothalamus, may be more effective at stimulating ovulation in anestrous ewes than a continuous infusion.

Research also has been conducted to examine estradiol-induced luteolysis in the ewe when stimulated by frequent, pulsatile injections of GnRH (Adams et al., 1975). Ewes were either pretreated with estradiol-17β or vehicle and all ewes received GnRH on day 12 of the cycle. In all estrogen treatment groups, a significant decrease was observed in luteal progesterone levels; however, GnRH control ewes had increased luteal weights and luteal progesterone content. It was concluded that administering only GnRH to ewes over a 72 hour period was able to stimulate luteal function similar to that in day 10 untreated control ewes. These researchers also concluded that pulsatile administration of GnRH may be more effective at stimulating the ovarian responses.

In cattle, ovarian responses to repeated administration of GnRH were evaluated (Kinder et al., 1975). Treated heifers received 150 μg of GnRH at 4 hour intervals for 96 hours on days 16-19 of the estrous cycle. These heifers were observed to have an increased mean serum LH concentration as compared to control animals. The treated heifers also

exhibited increased peak LH levels during the first one-half of the treatment period as compared with the second one-half of the period. However, it was concluded that pulsatile-like administration of 150 μg of GnRH given at 4 hour intervals was not enough to stimulate ovarian function (Kinder et al., 1975).

A study conducted by Mee et al (1993), concluded that one injection of GnRH administered at estrus resulted in decreased LH secretions 1, 3, and 8 days after administration. However, progesterone concentrations were increased sooner after estrus and remained elevated significantly longer than those of control animals receiving saline. This may be due to a more complete luteinization of the cells of the follicle. Upon examining the morphology of the CL, researchers found that GnRH increased the percentage of large luteal cells (LLC) and decreased the percentage of small luteal cells (SLC; Mee et al., 1993). They found that the proportion of SLC decreased after the administration of GnRH when compared to the saline-treated control animals (69 vs. 86%, respectively). Although SLC contain the majority of LH receptors, LLC secrete much higher basal levels of progesterone (Fitz et al., 1982; Niswender et al., 1985). Mee et al. (1993) concluded that GnRH may promote the shift of SLC to LLC, thus increasing luteal concentration and secretion of progesterone.

However, there have also been conflicting observations in regard to the effects of GnRH on serum and luteal progesterone concentrations during the estrous cycle (Rodger and Stormshak, 1986; Slayden and Stormshak, 1990; Whitmore et al., 1996). When GnRH was administered to ewes on days 2 and 3 after estrus, mean plasma progesterone levels were not affected (Whitmore et al., 1996). When lower doses of GnRH were injected directly into the ovarian artery of ewes on day 2 after estrus, progesterone was reduced on days 7-11 (Slayden and Stormshak, 1990). The observed response to exogenous GnRH was apparently due to the release of LH. In a subsequent study, Slayden and Stormshak (1990) found that administration of LH on day 2 also suppressed progesterone secretion on days 6-8.

Gonadotropin-releasing hormone was also administered during various stages of the estrous cycle to determine its effects on serum LH and progesterone levels, as well as its effects on the duration of the cycle in heifers (Rodger and Stormshak, 1986). When GnRH was administered on days 2 or 10 after estrus, an increase in serum LH was observed, with peak release occurring 15 to 30 minutes after injection. Gonadotropin-releasing hormone administered on day 2 caused a decrease in progesterone levels beginning on day 8. Administration of GnRH on day 10 was also effective in causing decreased progesterone levels; however, it caused a transient

increase during a three-hour sampling period after administration. There was no effect of the treatment on the duration of the estrous cycle.

Rodger and Stormshak (1986) also observed that GnRH administered on day 2 after estrus resulted in decreased CL weight on day 8 but not on day 14. Administration of GnRH on day 2 also resulted in a decrease in luteal LH receptor concentrations on both days 8 and 14. These researchers concluded that GnRH-induced release of LH on days 2 or 10 may have caused a more rapid transformation of small to large luteal cells with a consequent reduction in the number of LH receptors and progesterone synthesis or secretion.

In a review, Niswender (1981) stated that the mechanisms involved in the regulation of ovarian progesterone secretion were regulated by LH. This can occur by the down-regulation of LH receptors after elevations of systemic levels of LH as well as the regulatory role of other hormones. Luteinizing hormone binds to its specific receptor on the plasma membrane resulting in the activation of adenylate cyclase and results in elevated intracellular levels of cyclic adenosine monophosphate (cAMP). Thus, enhanced cAMP results in elevated activity of protein kinase A and increased steroidogenesis. Protein kinase A directly influences the activity of cholesterol esterase, causing cleavage of cholesterol esters to allow for utilization of cholesterol as a substrate for steroid synthesis via the activity

of the cholesterol side chain cleavage complex, the presumed rate limiting step in steroidogenesis.

STATEMENT OF THE PROBLEM

Domestic livestock are a very important agricultural commodity, providing us with a valuable source of protein from the milk and meat they produce. The goal of livestock producers is to rear a high percentage of healthy offspring, therefore a thorough understanding of the animal's reproductive cycle is a crucial component for management practices. A better understanding of the interactions between reproductive hormones could result in the development of more efficient estrous synchronization programs and, hence, increased artificial insemination (AI) conception rates when AI is combined with these improved programs.

Many factors can influence the life span of the CL. Understanding the aspects that control luteal life span may provide insight to the factors that direct the regulation of the estrous cycle and reproductive functions in ruminant livestock species. An important aspect in understanding the life span of the CL includes the interactions of luteal OT and uterine $PGF_{2\alpha}$ during luteal regression, and the various other hormones that affect the synthesis and/or secretion of OT.

The complex interrelationships between luteal OT and progesterone, as well as luteal OT and $PGF_{2\alpha}$, have yet to be determined. It is unknown what role luteal OT may play during the follicular stage as well as during

early luteal development. The effects that luteal OT may have upon the uterus early in the luteal phase have also yet to be determined.

It has been observed that progesterone receptor mRNA and the receptor are present in the CL, and their presence coincides with the rise in luteal OT mRNA and OT production Therefore, it is conceivable that progesterone may be acting in an autocrine fashion to promote OT synthesis and/or secretion in the ovine and bovine CL. This premise is supported by the research of Voss and Fortune (1993) who reported that progesterone stimulated OT secretion by bovine granulosa cells during the later stages of a 5-day culture. In addition, Lioutas et al (1997) found that inhibition of progesterone binding with the use of antagonists greatly reduced OT secretion from granulosa cells *in vitro*.

The experiments of the present thesis were conducted to determine: 1) if treatment with a progesterone antagonist would have an effect on luteal oxytocin synthesis and/or secretion in ewes by decreasing the ability of progesterone to bind to its own receptors, 2) to determine if GnRH-stimulated release of LH would have an effect on luteal function, thus affecting luteal oxytocin synthesis and/or secretion.

MIFEPRISTONE (RU 486)-INDUCED CHANGES IN OVARIAN FUNCTION OF EWES

ABSTRACT

Experiments were conducted to determine whether endogenous progesterone regulates synthesis and/or secretion of luteal oxytocin (OT). In experiment 1, mature ewes (n=5 per group) were assigned randomly to control or mifepristone (RU 486) treatment groups. Ewes were injected twice daily s.c. with vehicle or 10 mg RU 486 from days 5-7 of the estrous cycle (estrus = day 0). On day 8, following an i.v. prostaglandin $F_{2\alpha}$ (250 μg cloprostenol) challenge, venous samples were collected at frequent intervals to determine plasma OT concentrations. Plasma OT in RU 486-treated animals did not differ significantly from those of the control animals ($P > 0.05$). In Experiment 2, ewes were injected s.c. daily with vehicle or 175 mg RU 486 from days 2-5 of the estrous cycle followed by a prostaglandin $F_{2\alpha}$ challenge on day 6. Four of five RU 486-treated ewes exhibited "split-estrus" (estrous behavior through 36 hours and again 84 to 108 hours after the onset of initial estrus). There was no significant difference in mean plasma OT or progesterone levels between treatment groups ($P > 0.05$). Mean mature corpora lutea (CL) weights of control and RU 486-treated ewes on day 6 did not differ (394.8 ± 28.8 vs. 319.5 ± 48.3

mg; $P > 0.05$). Mifepristone-treated ewes contained mature CL, new CL (2 of 4 ewes), and/or preovulatory follicles ($\geq$ 10 mm, 2 of 4 ewes). Average interestrous interval for RU 486-treated ewes was 9 days longer than that of control animals (26.2 $\pm$ 2.9 vs. 17 $\pm$ 0.5 days; $P < 0.025$).

INTRODUCTION

Oxytocin (OT) is classified as a peptide hormone, consisting of nine amino acids. The traditional concept is that this nonapeptide is produced by the hypothalamus, and is stored and secreted by the posterior pituitary upon stimulation. However, results of studies conducted during the last two decades have indicated that OT is synthesized and secreted by the corpus luteum (CL) of ruminants during the estrous cycle (Wathes and Swann, 1982; Flint et al., 1990).

It has been proposed that in the ruminant, uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and luteal OT presumably act in concert through a "double positive feedback loop" to promote regression of the CL (Roberts et al., 1976; Roberts and McCracken 1976; McCracken et al., 1996). Results of previous research have confirmed that the administration of $PGF_{2\alpha}$ to cows

and ewes causes a transient rise in plasma OT levels, which in some studies has been shown to be associated with the induction and promotion of luteal regression (Flint and Sheldrick, 1982; Wathes et al., 1983; Flint et al., 1990; Orwig et al., 1994a; Salli et al., 2000).

The OT gene is actively transcribed in the bovine and ovine CL (Ivell and Richter, 1984; Ivell et al., 1985; Jones and Flint, 1988). Although granulosa cells *in vivo* appear to contain a low level of OT messenger ribonucleic acid (mRNA), gene transcription is upregulated on the day of ovulation to yield 100-fold higher levels of mRNA by day 3 of the estrous cycle (Furuya et al., 1990). Interestingly, there has been no significant positive correlation determined between the level of OT mRNA and of the peptide within the CL (Fehr et al., 1987). Previous research has demonstrated that luteal concentrations of OT mRNA are maximal on day 3 of the cycle and then gradually decline over the course of the cycle (Ivell et al., 1985; Jones and Flint, 1988; Furuya et al., 1990). In contrast, maximal luteal production of OT in the cow and the ewe occurs by the midluteal phase of the cycle and then declines so that luteal concentrations of the nonapeptide are comparatively low by the time of luteolysis (Sheldrick and Flint, 1983; Abdelgadir et al., 1987). These data indicate a delay in the peak of OT production 3-6 days after the OT mRNA peak, which coincides with maximal synthesis of luteal progesterone (Fehr et al., 1987).

Progesterone receptor mRNA, as well as the receptor protein, have been identified in ovine preovulatory follicles and CL (Smith et al., 1995). When bovine granulosa cells were cultured with the progesterone antagonists mifepristone (RU 486) and onapristione, OT secretion was significantly reduced and this effect could only be reversed in the presence of progesterone (Lioutas et al., 1997).

Because luteal progesterone receptor mRNA and the receptor coincide with the rise in luteal OT mRNA and OT production, it is conceivable that progesterone may be acting in an autocrine fashion to promote OT synthesis and/or secretion in the ovine CL. This premise is supported by the research of Voss and Fortune (1993) who reported that progesterone stimulated OT secretion by bovine granulosa cells during the later stages of a 5-day culture. Therefore, the objective of this research was to determine if the antagonistic actions of RU 486 would lead to decreased luteal OT synthesis and/or secretion.

MATERIALS AND METHODS

Animals

Mature Polypay ewes exhibiting normal estrous cycles (17 $\pm$ 1 days) were assessed for estrous behavior twice daily with a vasectomized ram and the first day of observed estrus was designated as day 0 of the estrous cycle. Ewes were assigned randomly to treatments prior to the beginning of the study.

To collect luteal tissue from ewes on days 6 and 8 of the estrous cycle, the animals were anesthetized with an i.v. injection of 5% sodium pentothal (Abbott Laboratories, N. Chicago, IL) followed by maintenance of anesthesia by use of closed circuit inhalation of an oxygen-halothane mixture (Halocarbon Laboratories, River Edge, NJ). The reproductive organs were exposed through a midventral abdominal incision. Luteal tissues were enucleated from the ovaries and immediately stored on ice until weighed. Follicles (5 to 10 mm diameter) were measured and recorded. Intramuscular injections of Banamine (Schering-Plough Animal Health Corp., Union, NJ; 50 mg/ml) and Penicillin G Procaine (Butler Co., Columbus, OH; 300,000 units/ml) were administered immediately following surgery. All experimental procedures and protocols were reviewed and

performed in accordance with the institutional Animal Care and Use Committee guidelines at Oregon State University.

Experiment 1- Luteal Oxytocin Synthesis

Ewes (n=5) were injected s.c. with 10 mg RU 486 (Sigma Chemical Co., St. Louis, MO) dissolved in 10 ml of corn oil twice daily on days 5, 6, and 7 of the estrous cycle. Control ewes (n=5) were injected similarly with corn oil only. On day 8, ewes were challenged with an i.v. injection of prostaglandin $F_{2\alpha}$ (250 µg cloprostenol). Blood samples were collected at -15 and 0 min to establish basal secretion of OT and at 2.5, 5, 10, 15, 20, 30, and 40 min post-challenge. An additional blood sample was collected prior to the prostaglandin $F_{2\alpha}$ on day 8 to determine plasma progesterone levels. All blood samples were collected into 10 ml heparinized vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) followed by immediate addition of EDTA (0.5 M; 20 µl) and 1, 10 phenanthroline (5 mg/ml in ethanol; 10 µl) to block endogenous oxytocinase activity and then stored on ice. Blood samples were centrifuged (1650 x g) at 4˚C, and plasma was stored at -20˚C until assayed for OT.

Experiment 1 - Ovarian Morphology

To evaluate the effects of RU 486 on ovarian function and morphology, ewes were assigned randomly to two treatment groups: 1) Control (n=4), 2) RU 486 (n=4). Ewes received s.c. injections twice daily of either 10 mg of RU 486 dissolved in corn oil or 10 ml of corn oil alone. Ewes were injected on days 5, 6, and 7 of the estrous cycle. On day 8, control and treated animals were anesthetized and luteal tissue was collected as described above.

Experiment 2 - Luteal Oxytocin Synthesis

Ewes (n=5) were injected s.c. with 175 mg RU 486 (Sigma Chemical Co., St. Louis, MO) dissolved in 10 ml of corn oil on days 2, 3, 4, and 5 of the estrous cycle. Control ewes (n=5) were injected similarly with corn oil only. On day 6, ewes were challenged with an i.v. injection of $PGF_{2\alpha}$ (250 µg cloprostenol). Blood samples were collected at frequent intervals as in Exp. 1 up to 60 min post-challenge. One sample was collected prior to the prostaglandin $F_{2\alpha}$ administration to determine plasma progesterone levels. All blood samples were collected into 10 ml heparinized vacutainer tubes

(Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and samples collected for OT analysis were followed by immediate addition of EDTA and 1, 10 phenanthroline and then placed on ice for transport to the laboratory. Blood samples were centrifuged (1650 x g) at 4°C, and plasma was stored at -20°C until assayed for OT and progesterone.

Experiment 2 - Ovarian Morphology

To evaluate the effects of RU 486 on ovarian function and morphology, ewes were assigned randomly to two treatment groups: 1) Control (n=4), 2) RU 486 (n=4). Ewes received s.c. injections of either 175 mg of RU 486 dissolved in corn oil or 10 ml of corn oil only. Ewes were injected once daily on days 2, 3, 4, and 5 of the estrous cycle. On day 6, control and treated animals were anesthetized and luteal tissue was collected as described above.

Oxytocin Radioimmunoassay

Oxytocin was extracted from 1 ml of plasma and measured by RIA using methods adapted from Schams (1983) and Abdelgadir et al. (1987), using an OT antibody (1:7000) generously provided by Dr. Dieter Schams, Technical University of Munich, Germany. The mean extraction efficiency was 63% as determined by the addition of [^{3}H] OT (4,000 cpm/ 100 µl; 2200 Ci/mmol; New England Nuclear, Boston, MA). Plasma concentrations of OT determined by RIA were corrected for losses due to extraction. Plasma sample volumes used in the RIA were 100 µl/ tube. The sensitivity of the assay was 1 pg/ml. All samples were analyzed in three consecutive assays with an intra- and interassay coefficient of variation (CV) value of 2.78 and 7.02%, respectively.

Progesterone Radioimmunoassay

Plasma concentrations of progesterone were assayed by RIA as described by Koligan and Stormshak (1976). Plasma progesterone was extracted from 100 µl of plasma with benzene:hexane (1:2). To correct for procedural loss due to extraction, [1,2,6,7-^{3}H] progesterone (4000 cpm/100

μl; 44.5 Ci/mmol; New England Nuclear, Boston, MA) was added to a third tube containing an aliquot of plasma The mean extraction efficiency was 88%.

Extracted samples were quantified by RIA using the #337 anti-progesterone-11-BSA (1:2400) provided by G.D. Niswender, Colorado State University. All samples were analyzed in two consecutive assays with intra- and interassay CV values of 1.9 and 8.4%, respectively. The sensitivity of the assay was 10 pg/ml.

Statistical Analysis

Plasma concentrations of OT were analyzed by use of repeated measures analysis of variance. Data on luteal weights were analyzed by analysis of covariance using number of corpora lutea as the independent variable and plasma concentrations of progesterone were analyzed by one-way analysis of variance.

RESULTS

Experiment 1 - Luteal Oxytocin Synthesis

Treatment of ewes with 10 mg of RU 486 twice daily on days 5-7 of the estrous cycle did not cause a significant decrease in luteal OT levels from those of control ewes following $PGF_{2\alpha}$ challenge (Fig.1.). Mean plasma concentrations of progesterone prior to the $PGF_{2\alpha}$ challenge on day 8 of the estrous cycle did not differ significantly ($P = 0.14$) between RU 486-treated and control ewes (3.06 ± 0.27 vs. 2.16 ± 0.35 ng/ml, respectively).

Experiment 1 - Ovarian Morphology

Mean weights of mature CL in control and RU 486-treated ewes did not differ (372.6 ± 32.6 vs. 397.2 ± 27.4 mg, respectively; $P > 0.05$). After administration of prostaglandin $F_{2\alpha}$, all control ewes displayed estrus within 48 hours. However, only 3 of the 5 RU 486-treated ewes displayed estrus within 48 hours after prostaglandin $F_{2\alpha}$ administration; the remaining two ewes had not responded by 96 hours after administration of $PGF_{2\alpha}$.

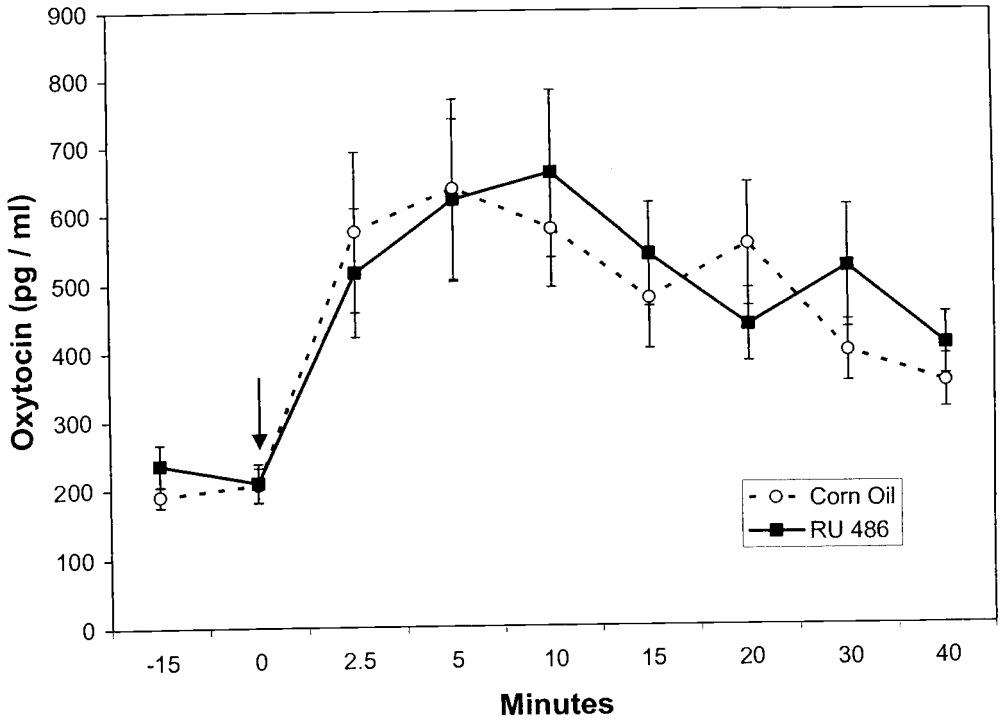

Figure 1. Mean (± SE) plasma concentrations of oxytocin following administration of prostaglandin $F_{2\alpha}$ on day 8 (0 min) of the estrous cycle in ewes treated with corn oil (n=5) or 10 mg of RU 486 (n=5) twice daily on days 5-7.

Experiment 2 - Luteal Oxytocin Synthesis

Treatment of ewes with 175 mg of RU 486 did not cause a significant decrease in luteal oxytocin levels from those of the control ewes (Fig. 2). Mean plasma concentrations of progesterone in RU 486-treated animals did not differ from those of the control animals (0.92 $\pm$ 0.04 vs. 0.94 $\pm$ 0.07 ng/ml; respectively). After the initiation of treatments, four of five RU 486-treated ewes exhibited another estrus, observed as early as 84 hours after the onset of the original estrus (Fig. 3.).

Experiment 2 - Ovarian Morphology

Mean weights of mature CL in control and RU 486-treated ewes did not differ (394.8 $\pm$ 28.8 vs. 319.5 $\pm$ 48.3 mg, respectively; $P > 0.05$). Mifepristone-treated ewes contained new CL (corpora hemorrhagica) and /or preovulatory follicles ($\geq$10 mm diameter) in addition to mature CL (Table 1). Average interestrous interval for RU 486-treated ewes was 9 days longer and was significantly different ($P < 0.025$) than that of the control animals (17 $\pm$ 0.5 vs. 26.2 $\pm$ 2.9 days; Table 1).

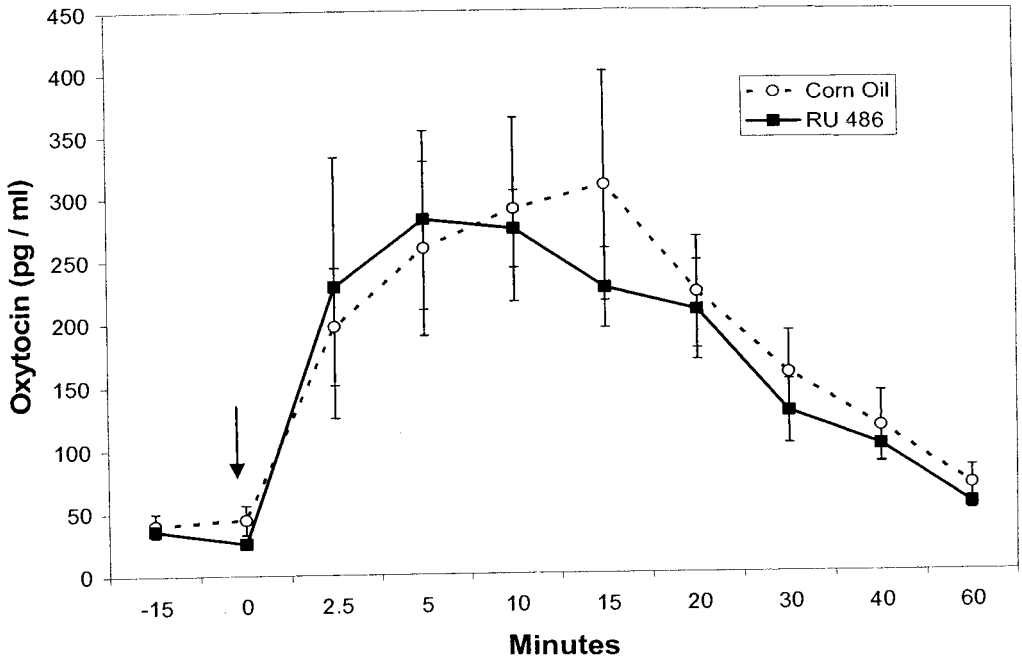

Figure 2. Mean (± SE) plasma concentrations of oxytocin following administration of prostaglandin $F_{2\alpha}$ on day 6 (0 min) of the estrous cycle in ewes treated with corn oil (n=5) or 175 mg RU 486 (n=5) on days 2-5.

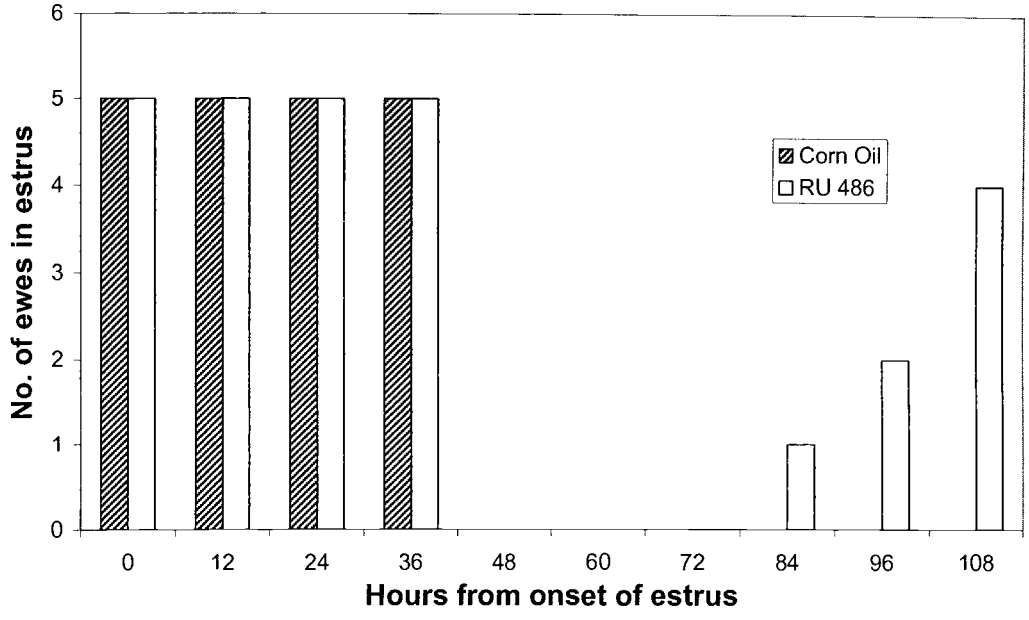

Figure 3. Effect of treatments on estrous behavior in ewes treated with corn oil (n=5) or 175 mg RU 486 (n=5) during the early luteal phase of the estrous cycle. Treatments were administered at 48 hours after the onset of the original estrus and continued through the treatment period. Between 84 and 108 hours after the initial injection of RU 486, four of five ewes exhibited estrus a second time.

Table 1. Ovarian characteristics of ewes treated with RU 486

Group [a]	No. of day 6 CL	Avg. (± SE) weight of day 6 CL (mg) [b]	No. of new CL	Avg. (± SE) follicle diameter (mm) [e]	Avg. (± SE) interestrous interval [f]
Control	6	394.8 ± 28.8 [c]	0	7 ± 0.26	17.0 ± 0.5 [c]
Treated	9	319.5 ± 48.3 [c]	5	9 ± 0.58	26.2 ± 2.9 [d]

[a] N = 4 ewes/group

[b] Data analyzed by analysis of covariance

[c,d] Means with a different superscript letter differ ($p < 0.025$)

[e] Two treated ewes had 4 follicles ≥ 10 mm diameter

[f] Interestrous interval was determined from the onset of the original observed estrus through the onset of the next normal estrous cycle

DISCUSSION

Administration of comparatively low and high dosages of RU 486 to ewes during the early to midluteal phase of the estrous cycle failed to affect OT synthesis and/or secretion and did not alter systemic concentrations of progesterone. Regardless of whether challenged on days 6 or 8 of the cycle, both control and treated ewes responded to exogenous $PGF_{2\alpha}$ with a maximal release of OT detected within 2.5 min after injections. The observed response to $PGF_{2\alpha}$ was similar to those previously reported by

Orwig et al. (1994a) and Salli et al. (2000). The systemic levels of luteal OT were substantially lower on day 6, during the early luteal phase, than on day 8 of the estrous cycle. Systemic OT levels after the administration of $PGF_{2\alpha}$ during the early and midluteal phase are also consistent with those values observed in the utero-ovarian venous blood of ewes within the same time period (Hooper et al., 1986).

Results of this study indicate that RU 486 administration while ewes are still in estrus and during metestrus causes a "split estrus". This split estrus was observed in four of five ewes receiving 175 mg RU 486 (within 84 hours after the initial observed estrus). This unique occurrence was not observed in ewes receiving the lower dose of RU 486 on days 5-7of the estrous cycle in Experiment 1. Likewise, split estrus was not reported by Morgan et al. (1993) who injected ewes with 2.5 mg/kg of RU 486 daily on days 5-8 of the estrous cycle. In a concurrent study in our laboratory, 150 mg of RU 486 administered to ewes at the onset of estrus, and 24, and 48 hours later, resulted in the same phenomenon of split estrus within 96 hours after onset of the original estrus (A. Wurst, unpublished data).

These observations of split estrus may be attributed to the antagonistic actions of RU 486 at the level of the hypothalamus. Mifepristone is known to be a potent antiprogestin in reproductive tissues, as well as the central nervous system (Philbert, 1984). In studies

conducted with female rat hypothalami, RU 486 bound with very high affinity, especially in the region of the preoptic nuclei (Pleim et al., 1990). Thus, it is conceivable that RU 486 may be binding to receptors located in the hypothalamus, blocking the negative feedback inhibition of progesterone, and encouraging the maturation of the next follicular wave. Campbell et al. (2000) concluded that blocking progesterone has major effects on the LH surge mechanism that can result in ovulation of post-estrus first wave follicles. This may be an explanation for the observed response of those ewes receiving 175 mg of RU 486 during the early luteal phase.

Ovarian morphology of ewes receiving 175 mg RU 486 revealed the presence of mature CL, new sites of ovulation, as well as preovulatory follicles occurring in various combinations on individual ovaries. It has been observed that ewes treated with RU 486 during the early luteal phase resulted in additional LH surges within hours after the initial ovulatory LH surge (Campbell et al., 2000). These researchers concluded that the subsequent LH surges resulted from normal levels of estradiol derived from preovulatory follicles, consistent with the findings of concomitant morphological studies.

As a result of the split estrus, the interestrous interval was extended in ewes receiving 175 mg of RU 486. The nine day extension in RU 486-treated ewes from the onset of the first observed estrus through the next normal estrous cycle is likely attributed to the development and ovulation of follicles that formed mature, functional CL. It was not until these newly formed CL regressed that the onset of the next estrous cycle could begin. Thus, the newly formed CL could have reset the "ovarian clock" for the estrous cycle. Although ewes did not display a split estrus in a study conducted by Morgan et al. (1993), by day 24 after the initial estrus RU 486-treated ewes still failed to return to estrus while the control animals had a normal cycle of 17 days in duration. These researchers attributed this failure to display estrus on inhibition of the progesterone receptors by RU 486, thus possibly interrupting the functional integrity of the ovarian-endometrial luteolytic axis by which progesterone-stimulates endometrial $PGF_{2\alpha}$ synthesis.

Administration of RU 486 during the early luteal phase of the estrous cycle appears to be a powerful antagonist at the level of the hypothalamus. It has been suggested that progesterone may act in an autocrine fashion to regulate its own synthesis, ultimately affecting its paracrine actions and regulation of other hormones (Rothchild, 1981; 1996). Although the highest dosage of RU 486 used in our study was apparently able to block the

negative feedback of progesterone at the level of the hypothalamo-hypophyseal axis, it was not able to interfere with luteal function. It is possible that the dosage was not sufficient to counteract the autocrine effects of progesterone at the level of the ovary. Future studies must be conducted to understand the actions of the preovulatory follicles and newly formed CL associated with the luteal phase of the estrous cycle when progesterone action is blocked early in the luteal phase.

SUPPRESSION OF LUTEAL OXYTOCIN AND PROGESTERONE SECRETION BY EXOGENOUS GONADOTROPIN-RELEASING HORMONE IN HEIFERS

ABSTRACT

An experiment was conducted to determine the effects of gonadotropin-releasing hormone (GnRH)-stimulated release of luteinizing hormone (LH) on luteal oxytocin (OT) and progesterone production in beef heifers. Ten heifers with normal estrous cycles were assigned randomly in equal numbers to a control and treatment group. On day 2 of the estrous cycle (estrus=day 0) heifers were injected with either physiological saline or 100 μg GnRH every 4 hours for 56 hours. Blood samples were collected 0 min pre- and 180 min post- GnRH challenge for progesterone analysis. Sixty hours after the initial injection of GnRH or saline, heifers were challenged with an i.v. injection of 500 μg prostaglandin $F_{2\alpha}$ (cloprostenol) and blood was collected at frequent intervals for OT analysis. Luteal OT synthesis was suppressed ($P < 0.01$) in heifers receiving repeated injections of GnRH compared to the saline-treated control animals. Progesterone secretion was significantly greater in saline-treated animals compared to GnRH-treated animals pre- and post-challenge (1.0 ± 0.06 vs.

0.93 ± 0.11 ng/ml and 1.16 ± 0.05 vs. 0.96 ± 0.13 ng/ml, respectively; $P < 0.05$).

INTRODUCTION

In the developing bovine corpus luteum (CL) transcription of the oxytocin (OT) gene increases up to about day 3 of the estrous cycle with maximal concentration of the nonapeptide achieved shortly thereafter (Furuya et al., 1990; Ivell and Richter, 1984; Jones and Flint, 1988). Luteinizing hormone (LH) appears to be the primary stimulation for OT production by granulosa cells of preovulatory follicles (Voss and Fortune, 1991; 1992), but little is known about the factor(s) regulating the synthesis of OT during the early luteal phase of the cycle. Because LH is luteotropic in the cow (Wiltbank et al., 1961; Simmons and Hansel, 1964), it might be anticipated that this gonadotropin would continue to stimulate OT production by the developing CL. However, existing experimental evidence suggests that brief exposure of the developing bovine or ovine CL to an excess of LH attenuates subsequent luteal function (Rodger and Stormshak, 1986; Slayden and Stormshak, 1990; Whitmore et al., 1996).

Although treatment with gonadotropin-releasing hormone (GnRH) to induce the release of LH, or administration of the gonadotropin, reduced the subsequent production of progesterone, the CL had a normal life span as judged by the interestrous interval.

Luteinizing hormone may act indirectly via the small luteal cells (SLC) to impact the function of the large luteal cells (LLC) because only the former cells are endowed with a majority of the LH receptors (Fitz et al., 1982). This mode of action of LH is supported by the data of Del Vecchio et al. (1994) who reported that bovine SLC are able to inhibit progesterone synthesis by perfused LLC. Apparently, the SLC produce an inhibitory substance that acts in a paracrine fashion to suppress LLC function.

It has been demonstrated that sequential injections of GnRH administered to beef cows during the late stages of the cycle results in a diminished release of LH with each injection (Kinder et al., 1975). On this basis, it was hypothesized that subjecting cows to such a treatment regimen of GnRH injections might have a detrimental effect on luteal function. Therefore, the present experiment was conducted to determine whether sequential injections of GnRH into beef heifers during the formation of the CL would alter secretion of luteal OT and progesterone.

MATERIALS AND METHODS

Nulliparous beef heifers were observed for estrus twice daily with a vasectomized bull. Heifers with estrous cycles of normal duration (20 ± 2 days) were assigned randomly in equal numbers to control (n=5) or treatment (n=5) groups. On day 2 of the estrous cycle, (day 0 = estrus) the jugular vein was catheterized with a 16 gauge, 3 ¼ inch indwelling catheter (Angiocath®, Becton Dickinson, Sandy, UT). The patency of the catheter was maintained by subsequent injections of heparinized-saline (40 IU/ml). Heifers received injections of either physiological saline or GnRH (100 μg gonadorelin acetate tetrahydrate, Cystorelin®, Merial Ltd., Essex, England) via the catheter every 4 hours for 56 hours. Samples were collected at 0 min pre- and 180 min post-GnRH challenge on day 4 (final GnRH injection) for progesterone analysis. All samples were collected into 10 ml vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Blood samples were allowed to clot and were stored at 4°C for 24 hours. Sera were separated by centrifugation (1650 x g) at 4°C for 10 min and stored at -20°C until analysis.

Sixty hours after the initial injection of GnRH or saline, heifers were challenged with an i.v. injection of prostaglandin $F_{2\alpha}$ (500 μg cloprostenol) and blood was collected at -15 and 0 min to establish basal secretion of OT

and at 5, 10, 15, 20, 30, 40, 50, and 60 min post-challenge. All samples were collected into 10 ml heparinized vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) followed by immediate addition of EDTA (0.5 M; 20 µl) and 1, 10 phenanthroline (5 mg/ml in ethanol; 10 µl) to block endogenous oxytocinase activity and then stored on ice. Blood samples were centrifuged (1650 x g) at 4˚C, and plasma was stored at -20˚C until analysis.

All experimental procedures and protocols were reviewed and performed in accordance with the institutional Animal Care and Use Committee guidelines at Oregon State University.

Oxytocin Radioimmunoassay

Oxytocin was extracted from 1 ml of plasma and measured by RIA using methods adapted from Schams (1983) and Abdelgadir *et al.* (1987), using an OT antibody (1:7000) generously provided by Dr. Dieter Schams, Technical University of Munich, Germany. The mean extraction efficiency was 69% as determined by the addition of [^{3}H] OT (4,000 cpm/ 100 µl; 2200 Ci/mmol; New England Nuclear, Boston, MA). Concentrations of OT in 100 µl of plasma sample determined by RIA were corrected for losses due to

extraction. The sensitivity of the assay was 1 pg/ml. All samples were analyzed in two consecutive assays with intra- and interassay coefficient of variation (CV) values of 2.13 and 3.50%, respectively.

Progesterone Radioimmunoassay

Plasma concentratons of progesterone were assayed by RIA as described by Koligan and Stormshak (1976). Plasma progesterone was extracted from 100 µl of plasma with benzene:hexane (1:2). To correct for procedural loss due to extraction, [1,2,6,7-^{3}H] progesterone (4000 cpm/100 µl; 44.5 Ci/mmol; New England Nuclear, Boston, MA) was added to a third tube containing an aliquot of plasma. The mean extraction efficiency was 88%. Extracted samples were quantified using the #337 anti-progesterone-11-BSA (1:2400) provided by G.D. Niswender, Colorado State University. All samples were analyzed in two consecutive assays with intra- and interassay CV values of 1.9 and 8.4%, respectively. The sensitivity of the assay was 10 pg/ml.

Statistical Analysis

Plasma concentrations of OT were analyzed by use of repeated measures analysis of variance. Data on plasma concentrations of progesterone were analyzed by analysis of variance for a 2x2 factorial arrangement of groups.

RESULTS

Repeated injections of GnRH significantly ($P < 0.01$) reduced plasma concentrations of luteal OT when compared to the saline-treated control animals (Fig. 4). Serum progesterone concentrations were significantly ($P < 0.05$) reduced in GnRH-treated animals compared to the saline-treated controls at 0 min and 180 min post-challenge (1.0 ± 0.06 vs. 0.93 ± 0.11 ng/ml and 1.16 ± 0.05 vs. 0.96 ± 0.13 ng/ml, respectively; Table 2)

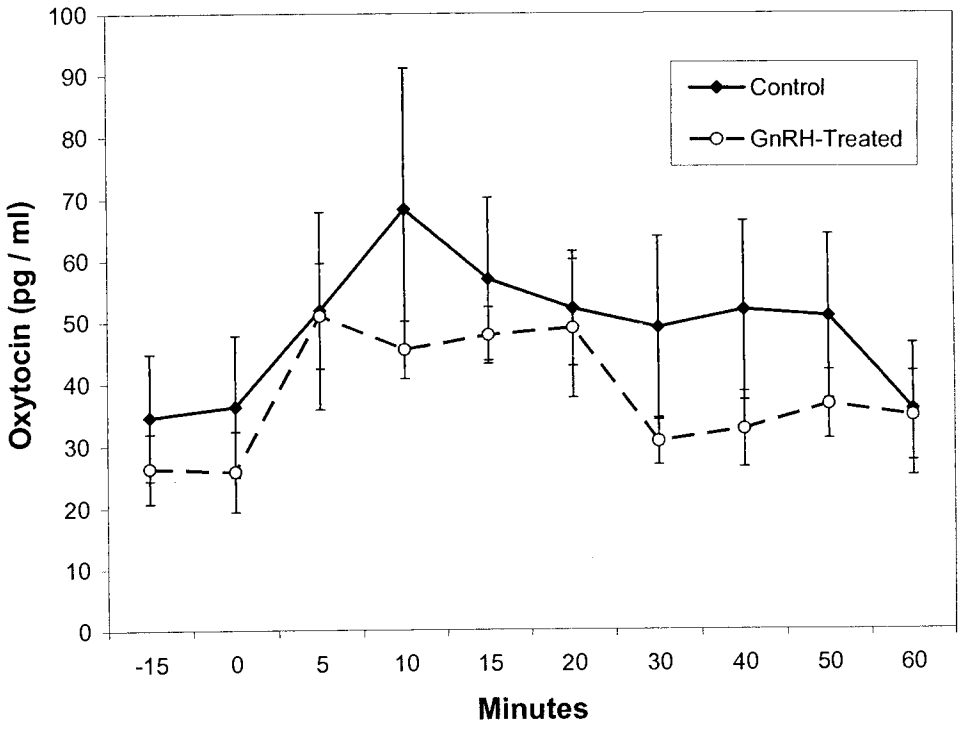

Figure 4. Mean (± SE) plasma concentrations of oxytocin following prostaglandin $F_{2\alpha}$ challenge (0 min) on day 4 of the estrous cycle in heifers treated with saline (n=5) or 100 μg GnRH (n=5) every 4 hours on days 2-4 of the estrous cycle.

Table 2. Mean (±SE) serum concentrations of progesterone in control and treated heifers

Group [a]	Time (min) 0	180
Control	1.00 ± 0.06 [b]	1.16 ± 0.05 [b]
Treated	0.93 ± 0.11 [c]	0.96 ± 0.13 [c]

[a] N = 5 heifers/group
[b,c] Means with different superscript letters differ ($p < 0.05$)

DISCUSSION

Results of the present experiment demonstrate that repeated injections of GnRH into beef heifers over a period of 4 days causes marked reductions in progesterone secretion and $PGF_{2\alpha}$-induced secretion of OT. It is unlikely that exogenous GnRH acted directly to alter luteal function because no GnRH receptors have been detected in the bovine follicle or CL (Brown and Reeves, 1983).

It is presumed that the initial injection of 100 μg GnRH caused a comparatively large release of LH and that each subsequent injection of GnRH resulted in a progressively diminished release of the gonadotropin.

Such a pituitary response to repeated injections of GnRH into cows and anestrous ewes has been reported by Kinder et al. (1975) and Chakraborty et al. (1974). In cattle, ovarian responses to repeated administration of 150 μg GnRH at 4 hour intervals were evaluated by Kinder et al. (1975). These heifers were observed to have an increased mean serum concentration of LH after the initial injection of GnRH which diminished over time with subsequent injections of GnRH. Exposure of the ovaries to successive releases of LH failed to affect their function. However, it should be noted that the repeated injections of GnRH were given during days 16 to 19 of the cycle when CL function is already waning.

The effect of the current treatment regimen on luteal function could be due to the excessive quantities of LH to which the developing CL was exposed. It has been reported by Rodger and Stormshak (1986) that a single injection of GnRH administered to beef heifers on day 2 of the cycle resulted in a significant reduction in luteal progesterone secretion. These latter investigators reported that induced release of LH resulted in a down-regulation of luteal LH receptors. A similar response to LH in the present experiment could have accounted, at least in part, for the reduction in progesterone secretion. It is also possible that exposure of the developing CL to the excessive amounts of systemic LH may have altered the ratio of SLC to LLC as the CL developed. Mee et al. (1993) found that an injection

of GnRH at estrus shifted the ratio of these cell types resulting in the formation of more LLC. It is unlikely that this phenomenon occurred in our experiment because LLC, although fewer in number then the SLC, produce the majority of progesterone (Fitz et al., 1982; Niswender et al., 1985) and are the source of OT (Fields and Fields, 1986; Theodosis et al., 1986)

It is possible that initial stimulations of SLC by LH caused these cells to produce a paracrine factor that acted on the LLC to impair their function. Del Vecchio et al. (1994) reported that the perfusate of bovine SLC suppressed progesterone production of LLC. It is also noteworthy that the CL is endowed with progesterone receptors (Smith et al., 1995). The precise role of progesterone receptors in regulating luteal function has yet to be established. Perhaps continued exposure of the developing CL to LH over a 4 day period affected the concentration and/or function of the progesterone receptors, and consequently the overall function of the gland.

Although luteal function was attenuated in heifers treated with GnRH, it did not result in an abbreviated estrous cycle (control, 19.2 $\pm$ 0.48 vs. treated, 19.5 $\pm$ 0.65 days). Thus, enough progesterone was being produced to maintain a normal feedback effect of the steroid on estrous behavior. Further studies need to be conducted to elucidate the exact mechanism by which LH affects luteal OT, progesterone, and progesterone receptor concentrations during development of the CL.

GENERAL CONCLUSIONS

The role of progesterone, in terms of regulating the uterine OTR, has been well established (Sheldrick and Flint, 1985; Vallet et al., 1990). However, the regulatory role (either autocrine or paracrine) progesterone may play within the ovary remains ambiguous. Rothchild (1981; 1996) has hypothesized that progesterone may act in an autocrine fashion to regulate its own synthesis, ultimately affecting its paracrine actions and regulation of other hormones. Therefore, the underlying theme of the studies presented was to attempt to determine some link between luteal OT synthesis and/or secretion and progesterone.

Various *in vitro* studies have provided evidence for a link between progesterone and luteal OT (Fehr et al., 1987; Chandrasekher and Fortune, 1990; Lioutas et al., 1997). However, results of our study utilizing the progesterone antagonist, RU 486, failed to affect luteal OT secretion in ewes. Although the highest dosage of RU 486 used in our study was apparently able to block the negative feedback of progesterone at the level of the hypothalamo-hypophyseal axis, it was not able to interfere with luteal function. It is possible that the dosage was not sufficient to counteract the autocrine effects of progesterone at the level of the ovary.

In contrast, heifers receiving repeated administration of GnRH during the early luteal phase resulted in a decline in progesterone and luteal OT secretion. Perhaps because the CL is endowed with progesterone receptors (Smith et al., 1995), continued exposure of the developing CL to LH over a 4 day period affected the concentration and/or function of the progesterone receptors, and consequently the overall function of the gland.

The question still remains, what link does progesterone play in regulating luteal OT? Future studies need to be conducted to delineate the exact role progesterone plays in regulating luteal function in the ruminant. Experiments designed to determine the effect of progesterone antagonists and LH-stimulation on progesterone receptor concentrations in the CL, nuclear and membrane alike, would prove useful. Such experiments may also help define the intracellular mechanisms that affect the synthesis of OT upon inhibition or down-regulation of progesterone receptors.

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