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Fredrick Stormshak

To determine whether membrane-related events may be involved in attenuated luteal function after gonadotropin-releasing hormone (GnRH) administration, corpora lutea (CL) were removed from 10 beef heifers on day 7 of the estrous cycle after i.v. injection of GnRH or saline on day 2 of the cycle. Luteal slices were incubated with saline (control), luteinizing hormone (LH) or 8-bromo-cAMP for 2 h. *In vivo* administration of GnRH reduced LH and cAMP-stimulated progesterone production by tissue ($p<0.01$), but basal progesterone production was not affected ($p>0.05$). Luteal adenylyl cyclase activity did not differ between saline and GnRH-treated animals ($p>0.05$). Results of this experiment suggested that GnRH-induced alteration of bovine luteal function may be due to an effect distal to the point of cAMP accumulation.

To explore further the effect of GnRH on luteal cell function, 10 heifers were injected with saline or GnRH and CL removed as above. Dissociated (mixed) and small luteal cells (SC) were cultured overnight, then incubated for 2 h with medium alone (control), LH or cAMP. *In vitro* treatment with LH and cAMP increased progesterone in the medium relative to controls ($p<0.01$),

however, there was no effect of GnRH injection on progesterone production ($p>0.05$) nor in the percentage of large cells (LC) present in the mixed cell cultures ($p=0.95$). It has been previously found that the ratio of LC to SC increases in GnRH-treated animals. Many LC can be ruptured during dissociation of the CL, and it is possible that this procedure altered the number of LC, such that any differences that may have existed between the saline and GnRH-exposed CL were minimized. These data suggest that differences in the LC to SC ratio may indeed account for attenuated luteal function after exposure to GnRH.

To examine if early administration of GnRH alters response of the CL to prostaglandin (PG) $F_{2\alpha}$, beef heifers were injected with saline or GnRH on day 2 of the cycle (n=4/group), then injected with $PGF_{2\alpha}$ on day 8 and the CL removed 60 min later. Blood samples were collected for oxytocin (OT) analysis at frequent intervals after $PGF_{2\alpha}$ injection and for progesterone at 0 and 60 min. Induction of the early response gene c-jun or release of OT by $PGF_{2\alpha}$ was not altered by GnRH injection ($p>0.05$). Injection of $PGF_{2\alpha}$ decreased serum progesterone by 60 min post-injection ($p<0.05$), but was also unaffected by GnRH ($p>0.05$). These data support the hypotheses that c-jun expression and OT release are involved in $PGF_{2\alpha}$-induced luteolysis, but early administration of GnRH did not affect these processes.

Cellular Mechanisms of Altered Bovine Luteal Function in Response to Exogenous Gonadotropin-Releasing Hormone

by

Jennifer Elaine Bertrand

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

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Jennifer Elaine Bertrand, Author

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
b	bovine
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic 3', 5'-adenosine monophosphate
cDNA	complementary DNA
CL	corpus luteum, corpora lutea
cpm	counts per minute
DAG	diacylglycerol
dbcAMP	dibutyryl cAMP
dCTP	deoxycytidine 5'-triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
h	hour
hCG	human chorionic gonadotropin
HDL	high density lipoprotein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
i.v.	intravenous
IFN	interferon
IP_3	inositol trisphosphate
ITS	insulin-transferrin-selenium
kb	kilobase
LC	large luteal cell
LDL	low density lipoprotein
LH	luteinizing hormone
LPDS	lipoprotein deficient serum
MAP	mitogen-activated protein
MEK	MAP kinase kinase
min	minute
MOPS	3-(N-morpholino) propane-sulfonic acid
mRNA	messenger RNA
MSH	melanocyte-stimulating hormone
NPY	neuropeptide Y
o	ovine
PCR	polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

PG	prostaglandin
PKA	protein kinase A, cAMP-dependent protein kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate (also called TPA)
PMSG	pregnant mare serum gonadotropin
POMC	pro-opiomelanocortin
RNA	ribonucleic acid
rRNA	ribosomal RNA
RSK-1	ribosomal S6 kinase
RT	reverse transcription
s.c.	subcutaneous
SC	small luteal cell
SCC	side chain cleavage
SDS	sodium dodecyl sulfate
sec	second
StAR	steroidogenic acute regulatory protein
TGFβ	transforming growth factor β
TP	trophoblast protein
TPA	tetradecanoylphorbol-13-acetate (also called PMA)
Tris	Tris[hydroxymethyl]aminomethane
TSH	thyroid-stimulating hormone
UV	ultraviolet

CELLULAR MECHANISMS OF ALTERED BOVINE LUTEAL FUNCTION IN RESPONSE TO EXOGENOUS GONADOTROPIN-RELEASING HORMONE

REVIEW OF THE LITERATURE

OVERVIEW OF THE ESTROUS CYCLE IN DOMESTIC ANIMALS

The estrous cycle in domestic female animals is characterized by several phases. The most obvious is the period of behavioral estrus, in which the female is sexually receptive to the male. Hormonally, this time is characterized by high systemic concentrations of estrogen, originating in the preovulatory follicle. Follicle-stimulating hormone (FSH) as well as luteinizing hormone (LH) from the anterior pituitary drive the synthesis of estradiol by the follicular cells. This ovarian estrogen also forms a positive feedback loop with LH, to eventually induce a spike release of LH that causes ovulation. In the cow, behavioral estrus lasts for only 12 to 24 h, with ovulation occurring approximately 12 h after the end of estrus. The cells of the ovulated follicle transform themselves into a endocrine structure known as the corpus luteum (CL). The stage of metestrus occurs during this short time of corpus luteum development. The luteal phase, which is comprised of metestrus and diestrus, is the dominant phase of the cycle, lasting 16 to 17 days of the 21 day cycle in the cow and sow. The luteal phase is also the dominant portion of the 16 to 17 day estrous cycle of the ewe and the 19 to 25 day cycle of the mare. During diestrus, the corpus luteum is fully developed and secretes increased quantities of progesterone to help establish and maintain early pregnancy if the ovum has been fertilized. If the animal is not pregnant, regression of the corpus luteum occurs and progesterone secretion

declines. Growing follicles can attain larger size and release more estrogen once progesterone levels decrease. Thus the period of follicular dominance, the follicular or proestrus phase, begins. Behavioral estrus follows this proestrus period. Ovulation occurs around the time of estrus. Ewes ovulate 24 to 30 h after the beginning of estrus, sows 35 to 45 h after the onset of estrus, mares approximately one to two days before the end of estrus and, as mentioned, cows 10 to 12 h after the end of estrus (Hafez, 1987).

While the cow has estrous cycles throughout the year, and only becomes acyclic in response to stressors such as extreme heat, some animals have naturally occurring periods of anestrus. Ewes and mares are known as "seasonal breeders" who cycle in the fall and spring, respectively. Thus ewes are called "short-day" breeders and mares are "long-day" breeders. Photoperiod is the critical regulator of this phenomenon. The sow can breed throughout the year, but goes through a period of lactational anestrus after giving birth.

HYPOTHALAMO-HYPOPHYSIAL AXIS

Hypothalamic and Pituitary Hormones

The hypothalamus and pituitary are perhaps the most important regulators of endocrine function in the body, and as such they control many essential and non-essential bodily systems, including reproductive functions. The hypothalamus lies below the thalamus and forms the walls and lower part of the third ventricle of the brain. It is the source of several peptide hormones that stimulate or inhibit the secretion of hormones from the anterior pituitary. For example, thyrotropin-releasing hormone, a three amino acid peptide,

stimulates the release of thyrotropin, or thyroid-stimulating hormone (TSH). The decapeptide gonadotropin-releasing hormone (GnRH) causes the secretion of LH and FSH from gonadotrope cells of the pituitary. Corticotropin-releasing hormone is responsible for stimulating the release of corticotropin (adrenocorticotropic hormone or ACTH). Somatocrinin is somatotropin (growth hormone)-releasing hormone. There is also some evidence for prolactin and melanocyte-stimulating hormone (MSH)-releasing factors. Hypothalamic hormones that inhibit pituitary hormone secretion also exist, such as somatostatin (somatotropin release-inhibiting factor), prolactin-inhibiting factor (most likely dopamine) and MSH release-inhibiting factor (Hadley, 1992).

The pituitary, also known as the hypophysis, is composed of two main parts or lobes, the adenohypophysis or anterior pituitary, and the neurohypophysis, or posterior pituitary. In many species, an intermediate lobe, considered part of the adenohypophysis also exists. The anterior pituitary is the site of production of several major hormones. Somatotropin and prolactin are single subunit proteins that possess numerous amino acid sequences in common and are members of the growth hormone family of proteins. They are also structurally related to placental lactogen, a hormone produced by the placenta of species such as human, rat, mice and sheep (Miller and Eberhardt, 1983; Slater et al., 1986). Somatotropin stimulates general somatic cell growth (Greep, 1974), including enhancement of amino acid incorporation into muscle protein and stimulation of extracellular collagen deposition (Isaksson et al., 1985; Hughes and Friesen, 1986). The effects of somatotropin can be due to direct action or can work through other chemical factors ("somatomedins") such as insulin-like growth factors. Prolactin, as its name implies, partly regulates mammary growth, development and lactation, although it has many other diverse functions (Jaffe, 1981).

The gonadotropins, LH and FSH, as well as TSH, are glycoproteins composed of two subunits, α and β. Within a species, the α subunit of the three hormones is identical, while the β subunit confers biological specificity (Boothby et al., 1981). The gonadotropins play important roles in control of reproduction, and their functions will be discussed further throughout this literature review. The target of action for TSH is the thyroid gland, which produces the metabolic regulatory hormones thyroxine and triiodothyronine. These hormones can influence many bodily functions. They are also considered "permissive" because they are required for the actions of other hormones in some cases. For example, their presence is needed with that of somatotropin for early growth and development (Green , 1987; Hays, 1988).

Other pituitary hormones are derived from the peptide precursor pro-opiomelanocortin (POMC). Within its structure are the amino acid sequences for ACTH and α-MSH. These sequences are similar, in fact α-MSH is comprised of the first 13 amino acids of ACTH; thus each hormone can stimulate the target tissues of the other. The major role of the 39 amino acid long ACTH is to stimulate steroidogenesis of glucocorticoids by the adrenal gland. Corticosteroids are important in intermediary metabolism and other body functions. The major source of α-MSH is actually the intermediate lobe. Several other products are also derived from POMC including γ-MSH, β-lipotropin and β-endorphin (Hadley, 1992).

Oxytocin and vasopressin are two hormones of the neurohypophysis. In actuality, these peptides are produced in neurons of the hypothalamus, and transported down the axons of these cells for storage in the posterior pituitary. Oxytocin, a nine amino acid long peptide, controls milk release from the mammary glands and contractions of the uterus, and may be important in maternal and sexual behaviors (Soloff et al., 1979; Pedersen and Prange, 1985,

Murphey et al., 1987). It is also found in the ovary where it has other functions. Vasopressin, also known as antidiuretic hormone, is important in water balance (Robertson et al., 1976).

Feedback Systems

"It used to be said that the anterior pituitary is the conductor of the endocrine orchestra; if that is so, then it is the hypothalamus that writes the score, in response to feedback from the audience." -F.J. Karsch

Feedback is a critical element of all biochemical systems. Both the hypothalamus and pituitary are important regulators of endocrine events in the body. However, they must communicate with each other as well as with target organs to function effectively. There are two direct routes of communication between the hypothalamus and pituitary. The hypothalamus and posterior pituitary are connected neurally, while the hypothalamo-hypophysial portal system is a vascular connection between the hypothalamus and the anterior pituitary. The first evidence for a "downward" flow in the portal vessels (from the hypothalamus to the pituitary) was provided by Wislocki and King (1936), and has subsequently been observed directly in a number of species (Everett, 1994). However, several researchers have shown that some retrograde flow may occur (Oliver et al., 1977; Page and Bergland, 1977; Bergland and Page, 1978). Whatever the mechanism, pituitary hormones do modulate secretion of their own releasing factors by "short-loop" feedback on the hypothalamus. Target organs affect the function of the pituitary and hypothalamus by "long-loop" feedback: their hormone products travel through the blood stream to affect secretion of stimulatory hormones from the hypothalamus and pituitary. While

both negative and positive feedback mechanisms exist, to respectively inhibit or further stimulate hormone production, negative feedback is much more common in biological systems (Hadley, 1992).

Regulation of GnRH Secretion

The structure and function of purified mammalian gonadotropin-releasing hormone has been known for almost 25 years (Schally et al., 1971). It was isolated from hypothalamic nuclei and structurally identified as a decapeptide with the amino acid sequence (pyro)Glutamate-Histidine-Tryptophan-Serine-Tyrosine-Glycine-Leucine-Arginine-Proline-Glycine-NH_2. As mentioned above, release of this hormone can be affected by several feedback mechanisms. A great deal of GnRH secretion is ultimately controlled via long-loop feedback by gonadal steroids, such as progesterone and estradiol. In general, progesterone is inhibitory to GnRH secretion, while estradiol is stimulatory. This fits in with the overview of the estrous cycle, in that estradiol positively feeds back on the hypothalamus and pituitary to allow increasing amounts of LH to be released so it can cause ovulation. During the luteal phase, only small pulses of LH, and thus GnRH, are needed to maintain the corpus luteum. While these steroids can act directly on GnRH neurons (Kordon and Drouva, 1990), they also modulate the actions of nearby neurons that release products that affect GnRH secretion (Kordon et al., 1994). For example, noradrenergic neurons are steroid-sensitive (Heritage et al., 1977), and their product, norepinephrine, can stimulate GnRH neurons to release GnRH. Neurons that produce neuropeptide Y (NPY) act in a similar manner. Estrogen has been shown to enhance the sensitivity of GnRH neurons to NPY and

norepinephrine, while progesterone treatment after estrogen-priming has not been found to affect the pulsatile release of these neuromodulators (Terasawa, 1994). Gamma aminobutyric acid and opioid neurons, on the other hand, are inhibitory to GnRH release but are also modulated by steroids (Flugge et al., 1986; Nikolarakis et al., 1986). Their products can act by inhibiting the noradrenergic and NPY neurons, and may also directly inhibit GnRH neurons (Leranth et al., 1985).

Role of GnRH in LH Secretion

Pulsatile secretion of GnRH is necessary for maintaining different aspects of gonadotrope secretory function, including regulation of GnRH receptors on the cells, expression of gonadotropin subunit genes, maintenance of cellular LH pools and release of the gonadotropins themselves (Belchetz et al., 1978; Clayton, 1982; Barkan et al., 1985; Haisenleder et al., 1991). For example, GnRH has been shown to increase mRNA for the β subunit of LH *in vitro* (Andrews et al., 1988). However, changes in GnRH pulse amplitude and frequency can have differential effects on up- and down-regulation of gonadotropin gene expression, and thus subsequent production and availability for secretion (Haisenleder et al., 1994).

As noted, the gonadotrope cells of the pituitary contain plasma membrane receptors for GnRH. Binding of GnRH to its receptor causes a cascade of events to occur, including Ca^{2+} mobilization and activation of protein kinase C (PKC), that ultimately induces the secretion of gonadotropins (Conn, 1994). One way that the effects of GnRH on these cells may be modulated is through changes in the number of pituitary GnRH receptors on their cell surfaces. During the

estrous cycle in several species, including rats, hamsters, ewes and cows, the maximum number of GnRH receptors is found just before the preovulatory LH surge (Clayton et al., 1980; Adams and Spies, 1981; Crowder and Nett, 1984; Nett et al., 1987). After the LH surge, the number of GnRH receptors decreases rapidly. The number of receptors present during pregnancy and lactation are also less than what is observed during the estrous cycle (Clayton et al., 1980; Marian et al., 1981). However, receptor number does not necessarily directly correlate to amount of LH released in response to GnRH (Young et al., 1985). For example, maximal LH secretion *in vitro* is found when receptors are only 20% saturated. Additionally, ewes can still fully respond to GnRH administration when 50% of the receptors are blocked with a GnRH antagonist (Wise et al., 1984). Whether this notion of "spare receptors" holds true for other functions of the gonadotrope (e.g., FSH release) is unknown (Conn, 1994).

THE FOLLICULAR PHASE

Stages of the Follicle

The mammalian ovary is a dynamic organ with changes to its structure occurring daily. Most of these changes are due to the growth and atresia of follicles. Unlike males, who produce sperm throughout their lives, females possess their entire supply of primordial follicles either before birth (primates, ruminants) or neonatally (rodents, rabbits; Fortune, 1994). Oocytes are stored within individual follicles through the development of the female to maturity. The stages of follicular development can be classified by several criteria, including oocyte morphology and size or the number of supporting granulosa

cells and their organization (Gore-Langton and Armstrong, 1994). All follicles begin as primordial or primary follicles. Primary follicles consist of a oocyte, arrested in prophase I of meiosis, surrounded by a single layer of flattened granulosa cells. These are the follicles that make up the resting pool of follicles in the ovary and are not under hormonal control. During the reproductively active years, follicles leave the resting pool gradually and continually and begin to grow. The signals that initiate this break from the resting phase, and the mechanisms to ensure gradual departure are unknown (Fortune, 1994). Secondary follicles are characterized by addition of granulosa cells by mitosis, with at least two layers of granulosa cells present, as well as increases in oocyte size. The zona pellucida, a glycoprotein matrix surrounding the oocyte, and the basal lamina, separating the granulosa cells from the rest of the ovary, are also evident. Tertiary (antral, Graafian) follicles are those that feature an antrum filled with follicular fluid with further increases in granulosa cell number. Thecal cells are also present outside of the basement membrane. Large antral follicles can be classified as nonovulatory or preovulatory to indicate their maturity (Gore-Langton and Armstrong, 1994).

In some species, such as rat, primate and pig, follicles of ovulatory size (dominant follicles) develop only during the follicular phase and thus are destined for ovulation. For example, ultrasonography during the human menstrual cycle has revealed that ovulatory-size follicles do not develop during the luteal phase, but that a group of growing follicles emerges during the early follicular phase and one of those follicles continues to grow through the late follicular phase (Pache et al., 1990). In the pig it also appears that follicles of ovulatory size do not develop during the luteal phase. On day 3, no follicles are larger than 4 mm, while on day 13 the largest are 3 to 6 mm. By day 16 of the cycle (late luteal phase), average follicle diameter is 4.8 mm and only one of six

gilts studied possessed large (6 to 9 mm) follicles. Ovulatory follicles are 7 to 11 mm at estrus (Parlow et al., 1964; Clark et al., 1982; Guthrie and Knudsen, 1984). During pregnancy or pseudopregnancy in the rat, follicles do not grow to ovulatory size until the last two to three days preceding the next estrus (Hirshfield, 1991).

In other species such as cattle and horses, recruitment, follicular selection, and dominance occur at regular intervals during the estrous cycle. However, only the dominant follicles present during the follicular phase ovulate. As mentioned above, why some follicles are recruited to develop and fewer still are selected for dominance is still unknown, although a slight rise in plasma concentration of FSH seems to precede follicular recruitment (Fortune, 1994). For example, in primates, basal concentrations of FSH are slightly higher at the beginning of the follicular phase in comparison to the late follicular phase or the luteal phase (Abraham et al., 1972; Goodman et al., 1977). In rats, a secondary surge of FSH on the day of estrus is closely followed by the recruitment of the next set of ovulatory follicles (Smith et al., 1975). In the cow, the secondary surge of FSH on the day of ovulation also precedes the first follicular wave of the estrous cycle (Dobson, 1978; Walters and Schallenberger, 1984), and small rises in FSH occur before the second and third follicular waves as well (Adams et al., 1992). Additionally, ablation (in rats) or delay (in cows) of follicular recruitment after ovulation occurred when injected follicular fluid (containing inhibin) blocked the secondary FSH surge on the day of estrus (Grady et al., 1982; Turzillo and Fortune, 1990).

Ultrasonography has provided a useful tool for physically studying the recruitment and growth of follicles during the estrous cycle in species such as the cow. Until recently, patterns of follicular development in cattle were deduced indirectly by cyclical changes in hormone profiles and by a variety of

experimental methods, such as inspection of the ovaries at slaughter, laparoscopic examination after dye marking and analysis of follicular fluid after destruction of follicles of selected sizes (reviewed by Fortune et al., 1988). Sirois and Fortune (1988) utilized ultrasonography to analyze patterns of follicular growth and regression throughout entire estrous cycles in cattle. They found that development of bovine follicles occurs in distinct, regular patterns. Heifers exhibited development of two or three follicular waves during an estrous cycle, with the three-wave pattern more commonly observed. A "wave" consists of emergence of a group of follicles ≥ 5 mm in diameter. Within several days, one follicle becomes larger than the rest and is considered the dominant follicle. In cycles with the three-wave pattern, waves began on days 2, 9 and 16 of the cycle, whereas in animals exhibiting a two-wave pattern they emerged on days 2 and 11. In animals with three follicular waves the average cycle length was 20.7 days. Thus the ovulatory follicle first emerged approximately 6 days prior to ovulation. Other laboratories have found similar results in patterns of follicular waves (Savio et al., 1988; Ginther et al., 1989), although Ginther et al. (1989) reported that the two-wave pattern was more prevalent and the three-wave pattern was the alternative. Basal concentrations of progesterone and gonadotropins, and length of the estrous cycle appear to be important in regulating the wave-like pattern of follicular development found in cattle (Sirois and Fortune, 1990; Fortune, 1993; Stock and Fortune, 1993).

Concentrations of progesterone also appear to regulate size and distribution of follicles in the ewe (Brand and de Jong, 1973; Dailey et al., 1982), possibly acting via LH release (Schrick et al., 1993). Unlike the cow, however, transrectal ultrasonography of developing follicles during the estrous cycle and early pregnancy in ewes has revealed that follicles are recruited to the gonadotropin-dependent pool in a continuous manner, rather than in a wave-

like pattern (Schrick et al., 1993). Follicular development did show two "peaks" of activity, both during periods of low progesterone (during the first 8 days of the cycle and during luteal regression). However, no dominant follicles were present during these peaks. The absence of a single dominant follicle allows for the presence of several follicles capable of ovulating, a necessity in this polyovular species.

Hormones Produced by the Follicle

Two-Cell Theory of Estradiol Synthesis

The steroid hormone estradiol is a primary product of the ovarian follicles. Estradiol is a necessary product because it is responsible for events such as estrous behavior and positive feedback with the hypothalamus and pituitary to release LH, which eventually causes ovulation. In species including cattle, sheep, pigs and rats, interaction between the two cell types of the follicle, the theca and granulosa cells, is necessary for estrogen biosynthesis. This theory was first formed by Falck (1959). In the "two-cell, two-gonadotropin" model, LH acts on the theca interna to stimulate the production of androgens from cholesterol. The enzyme 17α-hydroxylase/C17-20 lyase catalyzes the rate-limiting conversion of 17α-hydroxyprogesterone to androstendione in the theca cells. Granulosa cells do not express this enzyme and thus are incapable of androgen biosynthesis. However, androgens (androstendione and also testosterone) do diffuse across the basement membrane to the granulosa cells from the theca interna. Under the influence of FSH, granulosa cells aromatize

the androgen to estrogen. The aromatizing capacity of the preovulatory follicle almost completely resides within the granulosa cells (Hillier, 1994).

Oxytocin

Another important hormone produced by the follicle is oxytocin. Oxytocin was identified in preovulatory follicles of cows by Wathes et al. (1984) and Schams et al. (1985) and in ewes by Wathes et al. (1986). Aladin-Chandrasekher and Fortune (1990) found that oxytocin stimulated progesterone secretion from bovine granulosa cells shortly before the ovulatory LH surge, thus suggesting that oxytocin might be involved in regulating the follicular to luteal phase shift, from estradiol production to progesterone production. Voss and Fortune (1991) then examined oxytocin production from cells of preovulatory bovine follicles collected both before and after the endogenous LH/FSH surge. Oxytocin production by granulosa cells isolated before the LH/FSH surge was increased by the addition of LH or FSH to the cell culture medium. The predominant source of oxytocin is the granulosa cells because the theca produced little oxytocin when cultured alone, and the source of the oxytocin that was detected was considered to be contaminating granulosa cells. Oxytocin production by granulosa cells collected after the preovulatory LH/FSH surge was not altered by addition of gonadotropins, but the quantity of oxytocin produced was much greater than from those cells collected before the LH/FSH surge. This increase in oxytocin may be mediated by an increase in oxytocin/neurophysin-I mRNA in granulosa cells that occurs after the LH/FSH surge (Voss and Fortune, 1992). Voss and Fortune (1993) have also shown that estradiol has a biphasic effect on oxytocin secretion from follicular cells. Oxytocin production by granulosa cells was inhibited with high doses of

estradiol, but stimulated by low doses. These results are consistent with the results of Voss and Fortune (1991), in that oxytocin production by granulosa cells is lower before the endogenous LH/FSH surge, when concentrations of estradiol in follicular fluid are high (Fortune and Hansel, 1985; Fortune et al., 1988), and increased after the surge, when estradiol in follicular fluid is low. Thus the pattern of oxytocin secretion by the granulosa cells throughout the follicular phase suggests a role for oxytocin in regulation of luteinization and/or ovulatory events.

Ovulation

Ovulation involves a complex set of processes, that will be discussed briefly here, and has been described as an inflammatory reaction (Espey, 1980). It encompasses not only follicular rupture and release of the ovum, but also the events leading up to this biological "volcanic eruption" (Hill et al., 1935). The ovulatory process is initiated when the follicular tissue is stimulated by a surge of pituitary gonadotropins. Both LH and FSH have been recognized as being important in ovulation (Nalbandov et al., 1973), and under normal conditions they probably act together to initiate ovulation (Espey and Lipner, 1994). However, under experimental conditions, LH (Hisaw, 1947), hCG (McFarland, et al., 1989) or FSH alone (Schenken et al., 1984; Armstrong and Opavsky, 1988) can induce ovulation. But ovulation only occurs in mature follicles that contain adequate concentrations of LH receptors (Richards and Hedin, 1988; McFarland et al., 1989; Leung and Steele, 1992). Steroid synthesis by the follicle is increased after exposure to LH. Progesterone synthesis seems especially important in ovulation. For example, a progesterone synthesis inhibitor, isoxazol, was shown

to block ovulation in the ewe (Murdoch et al., 1986). The progesterone receptor antagonist RU 486 also partially blocked ovulation in rats that were treated with hCG to cause ovulation (Tsafriri et al., 1987).

Ovarian steroids and LH seem to synergize in stimulating follicular plasminogen activator (PA) secretion. Plasminogen activator is known to increase in preovulatory follicles (Beers, 1975; Beers and Strickland, 1978), and plasmin itself, the product of PA action on plasminogen, can decrease the tensile strength of the preovulatory follicle wall (Beers, 1975). It is thought plasmin acts by activating collagenase to initiate proteolytic processes that end with follicular rupture (Beers and Strickland, 1978). Prostaglandins may also be important players in the ovulatory process. Inhibition of prostaglandin synthesis by treatment with indomethacin, a cyclooxygenase inhibitor and nonsteroidal anti-inflammatory agent, has been shown to prevent ovulation in a number of species, including rodents, sheep, pigs and cows (Armstrong and Grinwich, 1972; O'Grady et al., 1972; Ainsworth et al., 1979; Downs and Longo, 1982; DeSilvia and Reeves, 1985; Murdoch and McCormick, 1991). The preovulatory LH/FSH surge causes a local increase in prostaglandin (PG) $F_{2\alpha}$ and PGE_2. However, the actual role for these prostanoids in ovulation is not clear, as some studies have indicated that they are involved in proteolytic enzyme activation (PA and/or collagenase), while others have found they have little effect (Murdoch et al., 1986; Murdoch and McCormick, 1991; Reich et al., 1991).

The preovulatory surge of gonadotropins ends because the pituitary becomes refractive to GnRH (Chakraborty et al., 1974; Kesner and Convey, 1982), not because of depletion of gonadotropins in the pituitary (Convey et al., 1981). Timing of ovulation varies among species. Ovulation occurs in ewes 24 to 30 h after the beginning of estrus, in sows 35 to 45 h after the onset of estrus, in mares

approximately one to two days before the end of estrus and in cows 10 to 12 h after the end of estrus (Hafez, 1987).

THE LUTEAL PHASE

Luteinization

Luteinization encompasses a series of events that morphologically and biochemically transform the cells of the ovulated follicle into the endocrine structure known as the corpus luteum. While this process truly begins before follicular rupture (Espey and Lipner, 1994), the actual breakdown of the basement membrane of the follicle allows blood vessels from the theca interna to invade the cavity of the ruptured follicle. This neovascularization is a process unique to the corpus luteum because it is the only place in the body in which angiogenesis occurs without unwanted tissue damage as the precipitating event. Within 48 h, the previously avascular granulosa cell layer has developed a complex network of new blood vessels (Koos, 1989). Some angiogenic factor secreted by the cells is thought to be responsible for the blood vessel formation. One potential candidate is basic fibroblast growth factor, which is produced by bovine granulosa cells (Neufeld et al., 1987) and corpus luteum (Gospodarowicz et al., 1985). Basic fibroblast growth factor derived from the CL has been shown to be angiogenic in a bioassay using vascularization of chick embryo choriallantoic membranes (Gospodarowicz et al., 1985). However, other growth or hormone factors may also be involved in neovascularization of the CL (Koos, 1989). At this early stage of development, the tissue is known as the corpus hemorrhagicum, indicative of the amount of blood vessels present. During this

stage there is also significant hypertrophy and hyperplasia of theca cells (O'Shea et al., 1980).

As luteinization progresses, the theca cells migrate into the follicular cavity and become dispersed among the luteinizing granulosa cells. Within a few hours of ovulation, granulosa cells accumulate smooth endoplasmic reticulum, containing 3β-hydroxysteroid dehydrogenase activity, and rounded mitochondria, characteristics of steroid secreting cells. These changes correlate with the initial rise in circulating progesterone (Niswender and Nett, 1994). Additionally, in response to the preovulatory surge of LH, cytochrome P450 side chain cleavage (SCC) enzyme activity increases, with an associated decrease in 17α-hydroxylase P450 activity (Rodgers et al., 1986, 1987; Lauber et al., 1991). These changes allow increased formation of pregnenolone from cholesterol, with subsequent creation of progesterone as the final product. Corpora lutea of some species, such as primates, do retain the ability to produce estradiol, although the follicular to luteal transition does entail a temporary decline in estrogen production compared to that of the preovulatory follicle (Hillier, 1994).

Changes in the populations of cell-surface gonadotropin receptors also occur during luteinization. Receptors for FSH disappear from granulosa-derived cells following ovulation, most likely due to internalization of receptor-hormone complexes and reduced gene expression (Nakamura et al., 1991). However, FSH receptors have been found on CL from cows (Manns et al., 1984) and hamsters (Oxberry and Greenwald, 1982). After the preovulatory LH surge, LH receptors are down-regulated, again probably due to internalization and reduced gene expression (Segaloff et al., 1990; LaPolt et al., 1990; Nakamura et al., 1991). However, expression of these receptors is subsequently enhanced approximately 48 h after exposure to gonadotropins (Braden et al., 1994).

Growth of the corpus luteum occurs quickly during its development. In the bovine, the CL rapidly increases in weight and progesterone content from days 3 to 12 of the cycle; these characteristics then remain relatively constant until day 16, when regression begins. In the ewe and the sow, both progesterone content and weight of the CL increase rapidly from days 2 to 8 and remain constant until day 15, when regression commences (Erb et al., 1971).

Corpus Luteum Structure and Function

Large and Small Luteal Cells

At least two distinct luteal cell types are found in the corpus luteum of many species such as cow (Ursely and Leymarie, 1979 ; Koos and Hansel, 1981), sow (Corner, 1919; Lemon and Loir, 1977), ewe (O'Shea et al., 1979), dog (Abel et al., 1975), rhesus monkey (Gulyas et al., 1979), human (Crisp et al., 1970) and rat (Wilkinson et al., 1976). Koos and Hansel (1981) first examined the morphology of large and small cells of the mid-cycle (days 11 to 13) bovine corpus luteum by transmission electron microscopy. They found that large luteal cells ($\geq$ 25 μm) contained a central, round nucleus with dispersed chromatin and a distinct nucleolus, two types of mitochondria, extensive rough endoplasmic reticulum, a highly convoluted cell surface and small (0.3 μm) electron-dense granules. These small granules were later identified to contain neurophysin (Fields and Fields, 1986) and oxytocin (Fields and Fields, 1986; Fields et al., 1992). The number of secretory granules has been shown to change during the estrous cycle, with the highest percentage of large cells containing secretory granules on day 7 (84%) and day 11 (64%), while lower percentages of large cells contained

granules on days 3 (3%), 17 (16%) and 19 (8%; Fields et al., 1992). Small luteal cells are described as having acentric, deeply-indented cup-shaped nuclei with heterochromatin lining the nuclear envelope, a relatively smooth surface, predominantly smooth endoplasmic reticulum, polymorphic mitochondria, and a large Golgi complex (Koos and Hansel, 1981). These authors noted that when cells were dispersed and clumps of small cells were observed, they appeared held together by extensively interdigitated microvilli.

Small and large luteal cells differ in ways that directly contribute to their functional capabilities. For example, the profile of hormone receptors found on their cell surfaces differ. Small ovine luteal cells have been shown to contain only low-affinity (nonspecific) binding sites for $PGF_{2\alpha}$ while large luteal cells contain a single class of high affinity binding sites with a K_d of 17.4±2.3 nM (Balapure et al., 1989). Fitz et al. (1982) reported that on a per cell basis, large luteal cells from superovulated ewes bound approximately 30 times more $PGF_{2\alpha}$ and ten times more PGE_2 than small luteal cells. This group also found that small luteal cells contained significantly more receptors per cell for LH/human chorionic gonadotropin (hCG) than did large cells. Other groups, however, have reported that in normally cycling ewes (Harrison et al., 1987), cows (Chegini et al., 1991) and rats (Nelson et al., 1992) the number of LH receptors on the two cell types was similar. However, the functionality of LH receptors on large cells is questionable, because *in vitro* exposure of large cells to LH does not cause an increase in cAMP or progesterone secretion as observed with small cells (Hoyer and Niswender, 1986). Still, prostaglandin $F_{2\alpha}$ and LH have been implicated in luteal regression and maintenance, respectively, thus understanding which hormone binds each cell type can aid in elucidation of specific large and small cell functions.

Origin of Luteal Cells

Because the follicle, from which the corpus luteum is derived, is composed of two distinct steroidogenic cell types, theca and granulosa cells, it is not surprising that two steroidogenic cell types exist in the corpus luteum. The percentages of cell types that comprise the corpus luteum and the origin of these cells, however, have come under some debate. Several histological studies on the formation of the bovine CL suggested that luteal cells are derived from both types of cells in the follicle, with the large luteal cells arising from the granulosa cells and the small luteal cells arising from the theca interna (McNutt, 1924; Foley and Greenstein, 1958; Gier and Marion, 1961; Donaldson and Hansel, 1965). However, it has also been suggested that in the ewe and the cow some large cells are derived from small luteal cells (Warbritton, 1934; Donaldson and Hansel, 1965; Fitz et al., 1982).

Alila and Hansel (1984) used monoclonal antibodies specific to granulosa and theca cell surface antigens to determine the contribution of the follicular cells to the bovine corpus luteum. They found that the granulosa cell antibody bound the large luteal cells, but percentage binding decreased as the corpus luteum aged. For example, percentage of large cells binding the granulosa antibody was 77±6% on days 4 to 6, 47.5±3% on days 10 to 12, and 30±3% on days 16-18. Binding to large cells from corpora lutea of pregnancy declined even further and was undetectable after 100 days gestation. Of the small luteal cells, 14% bound the granulosa cell antibody on days 4 to 6 of the cycle, and no binding was detected thereafter. The authors noted that these small cells resembled large cells in both nuclear and cytoplasmic features (Koos and Hansel, 1981) and suggested that these granulosa-derived small cells had not yet enlarged. However, most of the small cells bound the theca cell antibody

throughout the estrous cycle: binding was 70±4, 69±3 and 58±6% on days 4 to 6, 10 to 12 and 16 to 18, respectively. Binding of theca antibody to small cells was present in CL from pregnant cows, but binding did decline as gestation advanced. Binding of large luteal cells to the theca antibody actually increased during the estrous cycle. On days 4 to 6, 10±1.3% of large cells bound the antibody, while on days 10 to 12 the percentage binding increased to 46±3%. These investigators concluded that large luteal cells are initially derived from granulosa cells of the follicle, while small luteal cells are of thecal origin, but during the luteal life span small luteal cells differentiate into large luteal cells. It should be noted that some criticism of this study has been made (O'Shea et al., 1989) because enzymatically dispersed luteal cells were used for analysis, and losses of some of the large granulosa-derived cells could have occurred during this process (see below).

Composition of the Corpus Luteum

When using enzymatic dispersion techniques large cells generally do not account for more than 10% of the total luteal cell population (i.e., ≤10% large luteal cells, ≥ 90% small luteal cells) at any given time during the estrous cycle, including mid-cycle (Hansel et al., 1987, 1991). Small to large cell ratios of 20:1 to 40:1 (Hansel et al., 1987) and 10.2:1 (Weber et al., 1987) have been reported. However, it is recognized that cell numbers based on dispersion techniques are generally lower than morphometric techniques, because losses due to dispersion and separation of cells by size probably occur (Rodgers et al., 1984; O'Shea et al., 1989; Hansel et al., 1991). O'Shea et al. (1989) examined the numbers and types of cells in the bovine CL during the cycle by histological evaluation. They

determined, on a volume basis, that large luteal cells comprise a large part of the day 12 CL (40±7%), followed by small luteal cells (28±3%), endothelial cells and pericytes (13±2%), intercellular space (9.5±3%), fibrocytes (6±5%), other cell types/unidentifiable cells (2±0.6%) and vessel lumen space (1.2±0.4%). On a per cell basis, however, endothelial cells and pericytes, with a spherical cell diameter of approximately 11 μm, were found to be in the majority (52%), followed by small luteal cells (27%). Fibrocytes accounted for 10% of the cells, other/unidentified for 7.5%, while large luteal cells only comprised 3.5% of the total cells present. Thus, the small cell to large cell ratio in this study was 7.6:1. This study has come under some scrutiny (Hansel et al., 1991) because the animals were subjected to two doses of the luteolytic hormone $PGF_{2\alpha}$, which is a treatment known to reduce fertility (Smith et al., 1984) and alter luteal cell number and CL function (Hansen et al., 1987).

Lei et al. (1991), however, obtained somewhat similar results for percentages of luteal and nonluteal cells using animals that had been synchronized using a progesterone intrauterine device and one $PGF_{2\alpha}$ injection. They stated that fertility of these animals has been shown to be equal to that of normally cycling animals. In addition to mid-cycle corpora lutea, they examined CL from the early and late luteal phases as well as corpora albicantia and CL from pregnant animals. They found that more large cells were present during the late luteal phase (61% of total luteal cells) than at earlier times in the cycle (44 to 45% of total luteal cells). Thus the idea that small luteal cells develop into large luteal cells is somewhat supported. In mid-cycle corpora lutea, on a per cell basis, 60% of the cells were nonluteal cells and 40% were luteal cells. Of the luteal cells, 56% were small cells and 44% were large cells. In this case, then, the percentage of large cells out of the total number of cells would be higher (approximately 18%) than the 3.5% reported by O'Shea et al. (1989) and the small

cell to large cell ratio at mid-cycle would be 2.6:1. However, Lei et al. (1991) used a different size cutoff for distinguishing between large and small luteal cells than most others, as they considered a cell to be a large cell if it was ≥19.5 μ m. Generally, however, large cells are considered to be ≥22 or 23 μm and small cells approximately 15 to 22 μm. Thus differences in cell size interpretation might account for differences in percentage large cells reported, although their mean cell sizes did fall within the normally accepted ranges.

Parry et al. (1980) did not mention the use of $PGF_{2\alpha}$ for their study in which they used slaughterhouse CL from cows of known days of the cycle. Their morphometric results appear similar to those of O'Shea et al. (1989) as well, because on day 13 of the cycle the CL, on an area basis, consisted of 71% luteal cells, 7.3% blood vessels, 17.4% extracellular space and 4.3% other tissue. However, while these data seem comparable to those presented above on a volume basis, Parry et al. (1980) did not distinguish between large and small luteal cells in this instance. They did note that the large cells contained many electron dense granules as others have also reported.

Non-steroidogenic Cells in the Corpus Luteum

As noted above, the corpus luteum contains several types of non-steroidogenic, nonluteal cells. These cells may play roles in luteal function. Macrophages have been reported to be present in the CL of several species including rat (Bulmer, 1964), human (Gillim et al., 1969, Lei et al., 1991), mouse, (Kirsch et al., 1981) rabbit (Bagavandoss et al., 1988) and cow (Lobel and Levy, 1968). Macrophages are phagocytic cells and have been shown to contain remnants of luteal cells in degenerating CL (Paavola, 1979), although through their secretory abilities they have also been shown to potentially be both

luteotropic and luteolytic. Kirsch et al. (1981) reported that co-culture with macrophages promoted progesterone secretion by mouse luteal cells. The luteal cells did not respond to macrophage-conditioned media, implying that physical proximity of the two cell types was important for the observed stimulation. A role in luteolysis has been suggested as most likely, however, as few macrophages were found several days after ovulation, while many were found in regressing CL (Bagavandoss et al., 1988). These researchers found that macrophages are a likely source of tumor necrosis factor α in the CL. This cytokine could act through receptors found on numerous endothelial cells in the CL to aid in regression of this gland (Azmi and O'Shea, 1984; Bagavandoss et al., 1988).

Another type of white blood cell, the lymphocyte, is also present in the corpus luteum and is capable of secreting cytokines. Additionally, they may play a direct cytotoxic role and(or) recruit macrophages to the CL (Bagavandoss et al., 1988). Emi et al. (1991) suggested a luteotropic role for lymphocytes in the human corpus luteum. They found that progesterone production by granulosa cells luteinized *in vitro* increased when they were cultured in combination with lymphocytes or with lymphocyte-conditioned media. They determined that some secreted protein product of high molecular weight was responsible for this increase in steroidogenesis, and that its action was synergistic to that of gonadotropins. This group did not find a significant stimulatory effect of macrophages on the cells.

Other vascular elements, such as endothelial cells, are present in large numbers in the CL, due to the extensive neovascularization that occurs during luteal development. Endothelial cells are known to secrete PGI_2, (MacIntyre et al., 1978), which has been shown to have a luteotropic effect on mixed bovine luteal cells (Milvae and Hansel, 1980). Girsh et al. (1995) proposed that

endothelial cells may be able to establish intercellular contacts with large and small bovine luteal-like cells (granulosa and theca cells luteinized *in vitro*) in culture. Endothelial cells stimulated progesterone production from large cells, possibly via the action of PGI_2, suggesting a role for endothelial cells in maintenance of the CL. However, endothelial cells also produce and secrete the peptide endothelin (Yanagisawa et al., 1988), which has an inhibitory effect on steroidogenesis (Hison et al., 1991; Iwai et al., 1991) and luteinization (Iwai et al., 1991). Girsh et al. (1995) did postulate that endothelial cells may play a luteolytic role, because depression of hormone-stimulated progesterone secretion by $PGF_{2\alpha}$, found with intact luteal slices, was not observed with isolated small or large luteal-like cells. However, in co-cultures of large luteal cells with endothelial cells, $PGF_{2\alpha}$ significantly inhibited forskolin-stimulated progesterone production. Thus it is possible that endothelial cells, or some factor produced by them, could contribute to the effects of $PGF_{2\alpha}$ on luteal function.

Control of Progesterone Production

The steroid hormone progesterone is the considered to be the most important product of the corpus luteum, because without it pregnancy could not be established or maintained. It is also considered an important factor in maintaining function of the CL itself. Production of progesterone also seems to be differentially regulated in small and large luteal cells, and many factors can influence progesterone production both *in vivo* and *in vitro*. However, during the estrous cycle, secretion of progesterone and maintenance of the corpus luteum are ultimately dependent on LH in most species, including the cow, ewe and mare. Exceptions include laboratory animals, such as rats, which also rely

on prolactin as a luteotropic support (Niswender et al., 1985), the sow, which requires both LH and estrogen (Gardner et al., 1963; Cook et al., 1967) and the rabbit corpus luteum, which is dependent on estradiol (Braden et al., 1994).

Luteinizing hormone-stimulated progesterone production and the cAMP cascade

As mentioned previously, large luteal cells contain few LH receptors and therefore it is not surprising that large cells do not respond with greatly enhanced progesterone synthesis when incubated with LH. Ursely and Leymarie (1979) reported that bovine large cells could respond to LH with increased progesterone production; however, about 1000 times more LH was need in the large cell cultures to attain the same level of stimulation as with the small cells. Koos and Hansel (1981) found that bovine large luteal cells produce 20 times more progesterone than small luteal cells without the addition of LH. However, with LH, progesterone production by small cells increased dramatically (11-fold increase in 1 h, sixfold increase in 3 h), while large luteal cells only showed a twofold increase in progesterone production. Weber et al. (1987) found that prior to any incubation, progesterone content in bovine large luteal cells was seven times higher than in small luteal cells and 13 times higher after 3 h incubation. They also found that large cells did not respond to LH in culture with enhanced progesterone synthesis.

Fitz et al. (1982) studied how ovine luteal cells responded to the addition of LH. In their experiments, large cells produced about 20 times more progesterone than an equal number of small cells in the absence of hormonal stimulation. However, with the addition of LH or dibutyryl cAMP (dbcAMP), small cell progesterone production increased greatly, while the response of the large cells was only 10% that of the small cells. Thus large luteal cells produce

greater basal amounts of progesterone and may actually secrete the majority of progesterone produced by the CL of domestic animals. For example, some speculate that in the ewe, at least, the large cells are responsible for 80% of progesterone produced by the CL (Niswender et al., 1985).

In contrast to domestic animals, regulation of progesterone in large and small cells of the rat seems to be somewhat different. In one study, both large and small luteal cells from pregnant rats responded to dibutyryl cAMP with increased progesterone production; however, the large cells were responsive to a 10-fold lower dosage than the small cells (Smith and Sridaran, 1989). Also, large cells responded to increased substrate availability (25-hydroxycholesterol) with an increase in progesterone production. Thus the authors of this study suggested that large luteal cells are an important source of progesterone in the rat. However, Nelson et al. (1992) found that large and small cells from corpora lutea of pregnant rats were equally sensitive to LH stimulation of progesterone production.

Although they appear to be responsible for much of the progesterone production by the CL, mechanisms by which the large luteal cells of domestic animals produce this steroid are not well understood. The progesterone contribution of the small cells cannot be discounted, however, and they may be quite important in normal corpus luteum function including progesterone production, especially considering that LH is the primary luteotropic agent in domestic animals. In addition, how LH acts on the small luteal cell, which contains many LH receptors, to increase steroidogenesis is fairly well understood.

Luteinizing hormone exerts its influence by binding to its receptor on the cell surface. The LH/hCG receptor is a single polypeptide about 674 amino acids long. It is a member of the G protein-coupled receptor superfamily, and

contains seven hydrophobic transmembrane regions. The third cytoplasmic loop interacts with the stimulatory GTP-binding protein, G_s. The α subunit of this G protein can activate the membrane-bound enzyme adenylyl cyclase, which causes the conversion of ATP to cAMP in the cytoplasm. Cyclic AMP is an important "second messenger" molecule; it is the intracellular signal by which the action of LH is carried out (Leers-Sucheta and Stormshak, 1991). Cyclic AMP may have several important actions in the luteal cell. Primarily, it activates cAMP-dependent protein kinase A (PKA) by causing dissociation of the regulatory and catalytic subunits of the enzyme. This enzyme can then phosphorylate proteins such as cholesterol esterase, which frees cholesterol from intracellular stores. It may also be involved in activation of proteins that transport cholesterol into the mitochondria (Niswender and Nett, 1994).

Free cholesterol is transported into the inner membrane of the mitochondria, where it is acted upon by the cytochrome P450 side-chain cleavage (SCC) enzyme complex. The side-chain cleavage reaction is considered to be the rate-limiting step in steroidogenesis. However, it is actually the mobilization and transport of cholesterol to the enzyme, and not the catalytic action of the enzyme itself, that controls steroid synthesis (Waterman, 1995). Until recently, it was only known that some "labile protein factor" was required for cholesterol transport into the inner mitochondrial membrane. One recently identified candidate for the transport of cholesterol is steroidogenic acute regulatory protein (StAR), which has been cloned and expressed in MA-10 mouse Leydig tumor cells and has been shown to support steroidogenesis in the absence of hormonal stimulation (Clark et al., 1994). This protein does appear to be required for normal adrenal and gonadal steroidogenesis, although the exact mechanism by which it acts is not yet known (Lin et al., 1995). Immunocyto-chemical localization of this protein has shown that its expression is induced in

mitochondria of 30 to 40% of rat granulosa cells in culture after exposure to FSH for 24 h, with greater than 90% of cells positively staining after 48 h. The StAR protein expression was increased in the vast majority of MA-10 cells' mitochondria after 6 to 8 h treatment with 8-bromo-cAMP (Stocco et al., 1995) In the rabbit, presence of this mitochondrial protein in the corpus luteum appears to be regulated by estradiol, which is luteotropic in rabbits, and generally correlated with plasma concentrations of progesterone (Keyes et al., 1995). Once cholesterol is transported into the mitochondria of the luteal cell, cytochrome P450 SCC cleaves it to pregnenolone, which is subsequently transported out of the mitochondria and to the smooth endoplasmic reticulum. There, pregnenolone is converted to progesterone by the action of 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$ isomerase, and the progesterone can be secreted from the luteal cell (Niswender and Nett, 1994).

Cyclic AMP may have other actions in luteal cells beyond its involvement in progesterone production. For example, it seems to be necessary for the formation of luteal cells from granulosa cells (Richards et al., 1979) and allows for the maintenance of luteal cell morphology in cell cultures (Gospodarowicz and Gospodarowicz, 1975). Additionally, cAMP may be the intracellular messenger involved in the differentiation of small luteal cells into large luteal cells (Niswender et al., 1985).

Importance of lipoproteins

The substrate for progesterone synthesis in luteal cells, as noted, is cholesterol. Three main sources exist from which the luteal cell may obtain this precursor. The primary source is considered to be low-density lipoproteins and

high-density lipoproteins produced by the liver and carried in the circulation. Luteal cells contain receptors for these molecules, which allow them to bind to the cells. The lipoprotein-receptor complex is internalized and combined with lysosomes and the cholesterol is liberated. This free cholesterol can be stored in the cell as cholesterol esters in lipid droplets, which are then the secondary source of cholesterol for steroid biosynthesis by the cell. Additionally, luteal cells can make cholesterol within the cell directly from acetate, although this only occurs under certain conditions and is not preferred (Niswender and Nett, 1994).

The importance of lipoproteins in progesterone synthesis has been documented by several *in vitro* studies. Pate and Condon (1983) investigated the effects of both serum and isolated high- and low density lipoproteins (HDL, LDL) on mid-cycle bovine luteal cells cultured for 11 days. On all days of culture 10% serum inhibited LH-stimulated progesterone production by the cells and was found to be inhibitory at a point prior to the accumulation of cAMP, because cells in both serum-free and serum-supplemented media were able to respond to dbcAMP with increased production of progesterone. However, the actual mechanism for the inhibition of LH-stimulated progesterone production in serum-supplemented cultures is not known. Additionally, both dbcAMP-stimulated and basal progesterone production by cells in serum-supplemented media were greater than those of cells cultured under serum-free conditions. The authors postulated that these latter findings could be due to the presence of lipoproteins in serum, thus providing a potential substrate for steroidogenesis. To test this, they cultured bovine luteal cells with freshly isolated lipoproteins. During the first 24 to 72 h of culture LDL did not increase progesterone synthesis, but HDL stimulated a 1.5-fold increase over controls. In addition, neither HDL nor LDL inhibited LH-stimulated progesterone production. When

cells were not exposed to lipoproteins until day 3 of culture, addition of HDL and LDL for 2 to 4 more days greatly increased progesterone production without affecting LH response. This study showed that isolated serum lipoproteins can be a source of cholesterol substrate for progesterone synthesis by bovine luteal cells in culture. O'Shaughnessy and Wathes (1985) also found this to be the case, although they did not culture cells under serum-free conditions: bovine luteal cells were incubated in the presence of 10% whole serum, lipoprotein deficient serum (LPDS) or LPDS plus HDL or LDL. Both HDL and LDL in 10% LPDS medium increased basal and dbcAMP stimulated progesterone production. In this case the cells were more sensitive to LDL, although maximal response to the different lipoproteins did not differ. *In vivo*, HDL may actually be more important, since it is the major lipoprotein class present in bovine serum (Jonas, 1972; Raphael et al., 1973). Addition of HDL with a high cholesterol to protein ratio was found to stimulate progesterone production by bovine luteal cells more than supplementation with HDL having a low cholesterol to protein ratio (Carroll et al., 1992), again indicating that increased cholesterol substrate availability aids in progesterone synthesis with lipoprotein supplementation.

Other factors affecting progesterone production

There exist many other chemical factors that can modulate progesterone production by luteal cells both *in vivo* and *in vitro*. These include growth factors, cytokines and other locally produced hormones. There are probably many other factors that have yet to be discovered. However, many exist whose effects on progesterone production have been elucidated, and some of them will be discussed here.

In utilizing *in vitro* cell culture, care must be taken when exposing luteal cells to chemical agents not normally found *in vivo*. As shown above, addition of serum, often used in cell culture, can be detrimental to response of luteal cells to LH. Also, serum contains many unknown and varied amounts of hormones that can otherwise affect growth and(or) function of luteal cells in culture. However, serum-free medium does not seem to contain all the factors necessary for proper luteal cell function in culture. Addition of insulin or an insulin-transferrin-selenium (ITS) supplement to bovine luteal cells in serum-free culture has been shown to be essential for LH-stimulated progesterone production and aids in maintenance of basal progesterone synthesis (Poff et al., 1988). Commonly used antibiotics, such as gentamicin and penicillin-streptomycin do not seem to affect luteal cell function in culture, however, amphotericin-B, an antifungal agent, has been shown to decrease LH-stimulated progesterone production, although it did not affect basal progesterone production (Poff et al., 1988). Another common additive to cell culture medium is phenol red, a pH indicator. However, this compound has estrogenic activity, and its use is best avoided when culturing steroidogenic or steroid-responsive cells.

Of course, there are many factors found in the local chemical milieu of the CL *in vivo* that can affect its function. For example, the catecholamines dopamine and norepinephrine have been shown to be present in bovine luteal tissue on days 10 to 12 of the estrous cycle in ng/g quantities (Battista et al., 1989). Studies have also shown that catecholamines can stimulate progesterone production from the bovine CL both *in vivo* and *in vitro* (Auletta et al., 1972; Condon and Black, 1976; Battista and Condon, 1986; Battista et al., 1987, 1989; Kotwica et al., 1991). Additionally, it was shown that epinephrine-, norepinephrine-, isoproterenol- (a β-adrenergic receptor agonist; Condon and Black, 1976) or dopamine-stimulated (Battista et al., 1989) progesterone

production was inhibited by propranolol, a β-adrenergic receptor antagonist. At least dopamine-stimulated progesterone was not affected by an α-adrenergic receptor antagonist nor a dopamine-receptor antagonist (Battista et al., 1989). Thus it appears that stimulation of progesterone production by catecholamines is regulated through the β-adrenergic receptor. Norjavaara et al. (1989) showed that in rat CL the β_2 receptor subtype is found throughout pseudopregnancy, regardless of luteal age. In rabbit and pig, the β_1 receptor subtype seems to dominate. The exact mechanism through which β-receptor agonists exert their effects on progesterone production is still not clear, however. For example, there is no direct evidence that enzymes necessary for catecholamine production exist in the CL. Kotwica et al. (1991) found increases in plasma progesterone with infusion of a β-adrenomimetic drug, bamethan sulfate, and suggested, based on the work of Hsueh et al. (1983), that catecholamines can regulate steroidogenic enzymes in the CL. They also suggested that innervation of CL may be an important source of catecholamines.

Some growth factors, such as insulin-like growth factor, have also been implicated as having paracrine roles in the corpus luteum. Insulin-like growth factor-1 has been shown to increase progesterone secretion from luteal cells of the cow, rat and rabbit (McArdle and Holtorf, 1989; Dowd et al., 1990; Constantino et al., 1991; Talavera and Menon, 1991). Insulin itself has also been shown to increase progesterone release from the bovine CL (McArdle and Holtorf, 1989). Prostaglandins can be luteotropic; both PGE_2 and PGI_2 have been shown to increase progesterone from cow and sheep CL (Fitz et al., 1984; Alila et al., 1988). Oxytocin has also been implicated in maintenance of the corpus luteum. Miyamoto and Schams (1991) found that in microdialyzed bovine CL oxytocin stimulated an acute and dose-dependent release of progesterone. Additionally, infusion of an oxytocin antagonist blocked the oxytocin-

stimulated, but not LH-stimulated, progesterone production. In early and mid-cycle CL, pre-exposure to oxytocin increased LH-stimulated progesterone release, although when administered concomitantly no synergism between oxytocin and LH was evident. Stimulatory effects of oxytocin on progesterone production have also been shown with microdialyzed porcine CL (Jarry et al., 1990)

Luteolysis

The primary function of the corpus luteum is to aid in establishment and maintenance of pregnancy. If the ovum is not fertilized, however, it is important that progesterone production ceases and the CL regresses in a reasonable period of time so a new estrous cycle, and thus another chance for pregnancy, can occur. Luteolysis is a complex process that involves both functional and structural regression of the CL. Many factors, well-defined or still undiscovered, are involved in luteal regression, but it is generally agreed that prostaglandin $F_{2\alpha}$ is the hormone responsible for initiation of this process.

Roles of Prostaglandin $F_{2\alpha}$ and Oxytocin

In order for it to initiate luteolysis, $PGF_{2\alpha}$ of uterine origin must first make its way to the corpus luteum. It is thought that a counter-current mechanism exists by which $PGF_{2\alpha}$ in the uterine vein can cross into the ovarian artery. This vasculature is highly convoluted with the ovarian artery in close apposition to the uterine vein. McCracken et al. (1972) showed that during infusion of $[^3H]PGF_{2\alpha}$ into the uterine vein of ewes, labeled hormone could be

detected in the ovarian artery, with amounts increasing as the infusion progressed. Only a small amount of radioactive $PGF_{2\alpha}$ could be detected in the in the systemic blood, thus indicating that transfer from the uterine vein to the ovarian artery had occurred. In ewes greater than 99% of injected $PGF_{2\alpha}$ is metabolized in a single pass through the lungs, so the need for such a mechanism is evident. However, in the cow Davis et al. (1984) showed that 35.0±2.3% of injected $[^3H]PGF_{2\alpha}$ survived one passage through the lungs and 15.7±6.9% remained after three passages. Thus it is possible that $PGF_{2\alpha}$ also has a systemic effect in the cow.

Once it reaches the corpus luteum, $PGF_{2\alpha}$ can bind to its receptor on the large luteal cell (Fitz et al., 1983; Balapure et al., 1989). This receptor is coupled to the G_q protein, which interacts with a membrane-bound enzyme, phospholipase C (PLC; Smrcka et al., 1991). Phospholipase C can hydrolyze the membrane phospholipid phosphatidylinositol 4,5-bisphosphate, generating inositol trisphosphate (IP_3) and diacylglycerol (DAG; Leung et al., 1986; Davis et al., 1987). These compounds are considered second messengers in the PLC/protein kinase C (PKC) system. Diacylglycerol remains in the membrane where it can activate PKC, while IP_3 travels through the cytoplasm to the endoplasmic reticulum, where it causes the release of intracellular Ca^{2+}. Calcium can further activate PKC as well as other enzyme systems within the cell. Increased intracellular Ca^{2+} appears to mediate cytotoxic effects of $PGF_{2\alpha}$ in luteal cells, possibly by an apoptotic mechanism (Sawyer et al., 1990). Actions of PKC are thought to be ultimately responsible for the release of oxytocin from the large luteal cell. Activators of PKC have been shown to stimulate secretion of oxytocin from bovine luteal slices *in vitro* (Cosola-Smith et al., 1990) and membrane PKC activity has been correlated with plasma concentrations of oxytocin *in vivo* (Orwig et al., 1994). In the CL, PKC may act through

phosphorylation of the myristoylated alanine-rich C-kinase substrate (MARCKS; Orwig and Stormshak, 1994; Stormshak et al., 1995), a protein which is known to be involved in secretory events in other cells (Dunkley et al., 1986; Dunkley and Robinson, 1986).

Wathes and Swann (1982) first proposed that oxytocin could be found in the corpus luteum of ewes and cows. Subsequently, luteal concentrations of oxytocin have been found to be low in the early estrous cycle, maximal during mid-cycle and again low towards the end of the cycle (Sheldrick and Flint, 1983; Schams et al., 1985; Abdelgadir et al., 1987). Prostaglandin $F_{2\alpha}$ can cause the release of oxytocin, stored within secretory vesicles in large luteal cells (Fields and Fields, 1986; Theodosis et al., 1986), both *in vivo* (Schallenberger et al., 1984; Walters et al., 1984; Lamsa et al., 1989; Flint et al., 1990) and *in vitro* (Abdelgadir et al., 1987; Chegini and Rao, 1987; Jarry et al, 1992; Miyamoto et al., 1993). Thus it appears that $PGF_{2\alpha}$ is a primary regulator of luteal oxytocin secretion. At the end of the estrous cycle, both oxytocin and $PGF_{2\alpha}$ are secreted in an intermittent and concurrent pulsatile manner (Flint and Sheldrick, 1983; Walters et al., 1984). McCracken and Schramm (1983) proposed that a positive feedback loop exists between luteal oxytocin and uterine $PGF_{2\alpha}$ to promote luteolysis. However, factors that initiate and terminate this loop have not been determined, although the primary candidate is release of oxytocin from the posterior pituitary (Silvia et al., 1991).

Additional Contributors to Luteolysis

Of course, $PGF_{2\alpha}$ and oxytocin are not the only factors involved in regression of the corpus luteum. Initially, large luteal cells probably do respond to the luteolytic signal of $PGF_{2\alpha}$, but as mentioned previously, intercellular

communication between large and small luteal cells, as well as between luteal and nonluteal cells, is probably important for luteal regression to proceed. These include cells of the immune system, which may be actively involved in luteolysis (Pate, 1994). Increased numbers of eosinophils have been observed in CL of sheep that had been treated with $PGF_{2\alpha}$ (Murdoch, 1987). It has been suggested that the eosinophils might release cytotoxins that could injure luteal cells, cause changes in the cell membrane and(or) activate degradation of mRNA, resulting in luteal demise (Murdoch et al., 1988). Lymphocytes infiltrate the bovine CL starting on day 14 of the cycle (Lobel and Levy, 1968), before the onset of luteolysis. These cells produce lymphokines that attract and activate macrophages. Macrophages produce chemical products which may be involved in aiding luteal regression, such as tumor necrosis factor-α and interleukin-1 (Adashi et al., 1994). For example, interleukin-1β slightly suppresses progesterone production and dramatically increases prostaglandin synthesis by bovine luteal cells (Nothnick and Pate, 1990). Interestingly, structural luteolysis occurs less rapidly postpartum than at the end of the estrous cycle, and macrophages do not appear until day 15 postpartum (O'Shea and Wright, 1985).

Some cytokine-induced luteolytic events may be mediated by the formation of reactive oxygen species (Riley and Behrman, 1991). Reactive oxygen species and lipid peroxides are produced by the ovary, such as during the synthesis of prostaglandins (Behrman and Romero, 1991). The production of hydrogen peroxide, which evokes antigonadotropic and antisteroidogenic actions in ovarian cells, is stimulated by $PGF_{2\alpha}$ (Chance, 1979; Fridovich 1988; Lippman, 1989). Biological membranes are subject to attack by these oxidants because they contain high proportions of unesterified polyunsaturated fatty acids, which are especially sensitive to oxidative reactions. Orwig et al. (1992) suggested that the luteolytic effect of metabolites of the fatty acid

eicosapentaenoic acid that was tested in ewes may be mediated by production of reactive oxygen species, as at least one of the metabolites tested has been found to be a weak stimulator of superoxide anion production.

Maternal Recognition of Pregnancy

Maternal recognition of pregnancy is the critical time when an embryo must signal its presence to the mother in order to block regression of the corpus luteum and allow pregnancy to continue (Short, 1969). Maternal recognition of pregnancy occurs at days 16 to 17 in cows and days 12 to 13 in ewes (Niswender and Nett, 1994) and days 15 to 17 in female goats (Gnatek et al., 1989). This time corresponds with the period of blastocyst elongation. The principal signals for maternal recognition of pregnancy in these ruminant species have been identified as proteins released by the trophoectoderm (Roberts et al., 1990). These conceptus proteins have been characterized and structurally identified as members of the interferon (IFN) α_{II} subclass (Imakawa and Roberts, 1989). Interferons are small proteins with antiviral and antiproliferative activities, and the conceptus proteins have been shown to have these activities (Roberts, 1989). The proteins were previously known as ovine trophoblast protein-1 (oTP-1), bovine trophoblast protein-1 (bTP-1; Imakawa and Roberts, 1989) and caprine trophoblast protein-1 complex (cTP-1 complex; Gnatek et al., 1989), although the current designation is as species-specific IFN_{τ}.

Intrauterine administration of bTP-1 from days 15 to 21 in nonpregnant cows extended the interestrous interval from 19.5 to 26 days (Thatcher et al., 1989a). Similar experiments in cows and ewes using bovine recombinant (br) $IFN_{\alpha 1}$ also extended the time of progesterone production (Stewart et al., 1989;

Thatcher et al., 1989a). Intramuscular injections of brIFN$_{\alpha 1}$ from days 12 to 16 after mating resulted in a decrease in estimated early embryonic loss from 24% in control ewes to 8% in treated ewes (Nephew et al., 1990). Thus recombinant interferons may actually be of use to the animal industry to prevent early embryonic loss.

The mechanisms of action of trophoblast proteins are still under investigation. A specific receptor for oIFN$_{\tau}$ exists in the ovine endometrium and binding increases protein synthesis in the endometrium. However, oIFN$_{\tau}$ is not directly luteotropic as it did not increase progesterone production by luteal cells *in vitro* (Godkin et al., 1984). Rather, trophoblast proteins seem to function by blocking the production of uterine PGF$_{2\alpha}$ (Stewart et al., 1989). This effect occurs by action of IFN$_{\tau}$ on the endometrial luminal epithelium to prevent rapid increase in oxytocin receptors (Flint et al., 1989; Bazer, 1992), which normally occurs just prior to luteolysis (Ayad et al., 1991; Wathes and Hamon, 1993).

CONVERGENCE OF SIGNAL TRANSDUCTION SYSTEMS

The Mitogen-activated Protein Kinase Cascade

While it is certainly evident that both the protein kinase A and protein kinase C are important regulators of ovarian function, only recently have these signal transduction pathways been truly linked intracellularly. Both of these systems seem to converge at the newly elucidated mitogen-activated protein (MAP) kinase cascade, thus this series of phosphorylation events is a likely mediator of at least some of the actions of PKA and PKC. Three isoforms of MAP kinase are known to exist (Boulton et al., 1991) and at least two of them

require phosphorylation for kinase activity (Anderson et al., 1990; Serger et al., 1991). The enzyme is activated in response to a variety of stimuli including growth factors, insulin and other hormones.

The involvement of receptor tyrosine kinases, such as those of insulin and epidermal growth factor, in the activation of MAP kinase has only been recently fully understood. Through their plasma membrane receptors, these agents directly or indirectly activate the cytoplasmic protein known as Ras. Ras, now bound to GTP, can activate the serine/threonine protein kinase Raf, which in turn phosphorylates MEK, or MAP kinase kinase. Ras activation is not obligatory in the activation of Raf, however, because protein kinase C can also positively affect Raf directly. It appears that PKA can also directly affect Raf, although whether this regulation is in a positive or negative manner depends on cell type and other factors. After activation of MEK, MAP kinase itself is then phosphorylated and events affecting transcription can occur (O'Brien, 1994). For example, one way that MAP kinase appears to be able to regulate gene expression is through phosphorylation of transcription factors such as c-myc (Seth et al., 1991) and c-jun (Pulverer et al., 1991). MAP kinase can also activate other proteins such as ribosomal S6 kinase (RSK-1) and PHAS-1. Ribosomal S6 kinase can also phosphorylate transcription factors including serum response factor, Nur77, c-fos, c-jun and histone H3. Additionally, RSK-1 appears to be involved in cellular metabolism, playing a regulatory role in activation of glycogen synthesis (De Meyts et al., 1994). When phosphorylated, the protein PHAS-1 dissociates from eIF-4E, a factor that is required (in the unbound state) for initiation of protein synthesis (Lin et al., 1994).

Several preliminary studies have directly investigated this cascade in the female reproductive tract. Davis et al. (1995) examined the role of the MAP kinase cascade in the $PGF_{2\alpha}$-induced secretion of transforming growth factor β1

(TGFβ) from bovine luteal cells. They reported that the elements of the MAP kinase cascade were present in the bovine CL, including Raf-1, MEK, and three isoforms of MAP kinase. Both $PGF_{2\alpha}$ and phorbol myristate acetate (PMA), an activator of PKC, stimulated phosphorylation, and thus activation of the p44 form of MAP kinase, with subsequent dose-dependent release of TGFβ from the cells. Their data support the premise that the $PGF_{2\alpha}$ and PMA-mediated secretion of TGFβ ultimately depends on the MAP kinase activation of transcription factors, presumably including c-jun. Additionally, all elements of the MAP kinase cascade have been shown to be present in porcine granulosa, theca and luteal cells (Hildebrandt et al., 1995) and that hormonal stimulation of this cascade is possible at least in the porcine granulosa cell (Warren et al., 1995). Even though this pathway has only recently been elucidated, systems feeding into this pathway, such as hormonal activation of PKC and PKA have already been extensively studied. More recently some of the end points, such as induction of c-fos and c-jun in the female reproductive tract have come under investigation (see below).

Early Response Genes and their Products in the Reproductive Tract

C-jun is a nuclear protein that makes up part of the AP-1 transcription factor. AP-1 can be a homodimer of c-jun or a heterodimer of c-fos and c-jun. While the binding of the AP-1 homo- or heterodimer to its response element on DNA is the action that alters transcriptional activity of various genes, study of the regulation of the c-fos and c-jun genes is also of interest. They are called "early response genes", along with c-myc, because they respond quickly to hormonal and growth factor stimuli with increases in mRNA, thus subsequently

allowing additional protein expression of the transcription factors. "Delayed response genes" are those that take an hour or more to induce, require new protein synthesis for induction, and are likely stimulated by the products of the early response genes (Alberts et al., 1994).

The roles of several hormones on induction of early response genes in reproductive tissues have been studied. Several studies have focused on expression of c-jun in the uterus, a steroid-responsive portion of the reproductive tract. In ovariectomized rats, estradiol has been shown to induce c-jun gene expression (Cicatiello et al., 1993; Webb et al., 1993). In immature rat uterus, c-jun expression also increased after estradiol injection (Webb et al., 1990; Bigsby and Li, 1994), however when RNA from different uterine compartments was analyzed, c-jun was actually repressed in the uterine luminal epithelium (Bigsby and Li, 1994). In mature rats, a stronger induction of c-jun was found specifically in the stroma-myometrial tissue (Webb et al., 1990). These effects were also shown by Nephew et al. (1994) who found that expression of c-jun was increased by estrogen in uterine glands and myometrium of immature rats, but in mature rats the uterine glandular epithelium did not respond to estradiol with increased c-jun expression. Shelley et al. (1994) examined the induction of c-jun after tonic administration (via implants) of estradiol, progesterone or estradiol plus progesterone for 24 or 48 h. In this study, high dosage steroid treatment actually suppressed c-jun induction in the uterus by 48 h, although by 48 h expression in the ovaries and adrenals had increased. Thus, the role of expression of the early response genes in the uterus is somewhat equivocal, and their induction by steroids depends on timing and dosage of administration. At least in the immature rat uterus, no simple correlation exists between cellular proliferation and increased expression of the early response genes studied (Bigsby and Li, 1994). Additionally, the lack of maturational effects on c-jun

gene expression, and the differential response of the mature vs. immature rat uterus to estrogen in terms of cell proliferation (Nephew et al., 1994), seem to indicate unexplored roles for early response gene expression in the uterus.

Others studies have concentrated on early response gene induction in steroidogenic tissues. Both hCG and dbcAMP can transiently increase the levels of c-fos and c-myc mRNA in cultured mouse Leydig tumor cells (Czerwiec et al., 1989). In rat granulosa cells, FSH and dbcAMP increased c-fos and c-jun message in 30 min on both day 0 and day 2 of culture three- to fourfold, but LH, hCG and tetradecanoylphorbol-13- acetate (TPA) only markedly increased mRNA levels on day 2 of culture (Ness and Kasson, 1992). Khan et al. (1993) examined how $PGF_{2\alpha}$ could influence c-jun expression in the ovary, uterus and adrenal of pregnant mare serum gonadotropin (PMSG)-primed immature rats. Thirty minutes after a second injection of $PGF_{2\alpha}$, c-jun mRNA was increased in the ovary but not the uterus or adrenal. Induction of signal was stronger in corpora lutea on day 7 after ovulation than on day 3. Although not induced by $PGF_{2\alpha}$, c-jun mRNA was present in the adrenal, another steroid producing organ, but was barely detectable in the steroid-responsive uterus, whether from control or $PGF_{2\alpha}$-treated animals. In regard to its effect on the CL, the authors speculated that because the effect of $PGF_{2\alpha}$ was stronger in older than in younger CL, c-jun expression might be linked to the mode of action of $PGF_{2\alpha}$ in corpus luteum regression. In another study Khan et al. (1994) examined both c-fos and c-jun expression in CL from PMSG-primed immature rats on day 7 post-ovulation, at 15, 30 and 120 min after $PGF_{2\alpha}$ or PGE_2 injection. Injection of $PGF_{2\alpha}$ and PGE_2 induced a four- and twofold increase, respectively, in c-jun and c-fos expression. However, the effect on c-fos expression was transient and found only 15 min after prostaglandin injection, while message for c-jun remained elevated until 2 h post-injection. Expression of c-jun was threefold

higher in the $PGF_{2\alpha}$-treated animals than in those injected with PGE_2. From this study, the authors concluded that both luteotropic and luteolytic effects of these prostaglandins could involve activation of AP-1 responsive genes. All of the studies on hormonal regulation of the early response genes seem to indicate that they may be differentially regulated depending on cell type and stimulus, and that they could be very important in regulating nuclear events that ultimately control aspects of reproductive function.

ROLE OF GnRH IN OVARIAN FUNCTION

Mechanisms of Action

In both males and females, administration of pharmacological doses of GnRH or its agonists results in an increase in serum gonadotropins. Because gonadotropins are important regulators of gonadal function, researchers have sought to determine if exogenous GnRH can enhance fertility. In reality, it seems that GnRH has the potential to inhibit reproductive function by decreasing ovarian steroid production, decreasing numbers of gonadotropin receptors, inhibiting follicular development, maturation, and ovulation, delaying ovum transport and implantation, decreasing uterine growth and possibly terminating pregnancy (Hsueh and Jones, 1983).

At least three mechanisms, proposed by Hsueh and Jones (1983), can potentially account for the alteration of gonadal function by GnRH. One is that chronic stimulation or high doses of GnRH may cause a desensitization of the gonadotropes in the pituitary (Belchetz et al., 1978), causing decreased gonadotropin secretion and thus loss of gonadal function. This does not appear

to be true, however, at least for the effect of a somewhat acute administration of GnRH in the cow, because injection of 100 μg GnRH on both days 2 and 8 of the same estrous cycle caused a significant increase in secretion of LH on both days (Martin et al., 1990). Continuous administration of GnRH also does not appear to affect basal secretion of LH, nor responsiveness of the pituitary to bolus injections of GnRH. Lamming and McLeod (1988) continuously infused GnRH s.c. into cows for 14 days at 20 μg/hour. Within 48 h of the start of infusion, LH levels returned to pre-infusion concentrations. However, a bolus injection of 10 μg GnRH i.v. both before the start of infusions and on the fourteenth day of infusion caused an increase in plasma concentrations of LH, although the release after the second injection was significantly less than the release after the first. These results do indicate that the pituitary was still responsive to GnRH even after a long period of continuous exposure to GnRH.

A second possibility is that GnRH injection can stimulate the release of large quantities of LH, causing desensitization of gonadal cells to subsequent action of LH. In relation to the CL, a number of studies have shown that, at various times during the estrous cycle, GnRH injection can rapidly cause a significant increase in serum concentrations of LH in both the cow (Milvae et al., 1984; Rodger and Stormshak, 1986; Martin et al., 1990) and the ewe (Slayden and Stormshak, 1990, Whitmore, 1995). Rodger and Stormshak (1986) suggested that a GnRH-induced LH surge on day 2 of the cycle may indeed cause down-regulation of luteal LH receptors, because they found a significant decrease in receptor number on days 8 and 14 of the same cycle. It does not appear, however, that simply a decrease in luteal LH receptors fully explains alteration of luteal function by GnRH. Martin et al. (1990) showed that serum concentrations of progesterone did not differ between cows that had received injections of saline or GnRH on days 2 and 8 of the cycle, whereas Rodger and

Stormshak (1986) showed that a single GnRH injection on day 2 significantly reduced serum progesterone beginning on day 8 compared with saline controls. Thus it appears that in the double-injection study sufficient LH receptors were available to support the luteotropic action of this gonadotropin; the extra endogenous LH secreted in response to the exogenous GnRH on day 8 may have helped maintain luteal function.

Milvae et al. (1984) also reported that repeated injections of GnRH analogue on days 9 through 12 of the estrous cycle caused an increase in serum LH, at least initially, and actually increased serum progesterone during the same cycle when compared with saline-treated controls. However, serum concentrations of progesterone were depressed in the subsequent cycle. In the same study, repeated injections of native GnRH also initially caused significant increases in serum LH, although actual concentrations were slightly lower than with the analogue, but no effect was detected relative to serum progesterone. The authors postulated that this slightly lowered response of LH could cause the differences observed in serum concentrations of progesterone, or that GnRH analogue caused LH and FSH to be released in a different pattern than the native hormone. Thus timing and type of administration may affect actions of GnRH on the pituitary and almost certainly affect its actions on luteal function.

The third proposed mechanism is that GnRH acts directly on the ovary (or testis). This seems to be true in rats because high affinity binding sites for GnRH have been found in the ovaries (Clayton et al., 1979) and testis (Labrie et al., 1980) of this species. The receptor binding characteristics of these sites have been shown to be identical to those of the pituitary (Reeves et al., 1980). Clayton et al. (1979) additionally showed that these receptors are functional because basal progesterone production by isolated luteal cells decreased 25% when the cells were incubated with GnRH analogue. Stimulation of progesterone by low

levels of hCG was completely abolished in the presence of the analogue. Response of the cells to hCG could be attained, but the amount needed for 50% maximal stimulation was increased 25-fold in the presence of GnRH analogue. The effect was most likely mediated by the GnRH receptor because binding of hCG to the cells was not affected. Messenger RNA levels for the GnRH receptor in rat ovaries has also been recently examined (Whitelaw et al., 1995). Expression of GnRH receptor in granulosa cells appeared to be individually regulated for each follicle, and was present in the corpus luteum and atretic follicles.

In other species, it is not clear if GnRH can act directly on the ovary. Brown and Reeves (1983) showed that GnRH binding sites were not present on follicles or corpora lutea of cows, ewes or sows, using rat ovaries as well as pituitaries from these animals as positive controls. Other researchers were unable to demonstrate binding of GnRH to gonadal tissue of other species, such as monkey (Asch et al., 1981), human (Clayton and Huhtaniemi, 1982) and mouse (Hunter et al., 1982). Recently, however, GnRH receptor mRNA has been found to be expressed in human granulosa cells in culture, using reverse-transcription polymerase chain reaction (RT-PCR), a method that can detect very low levels of transcript (Peng et al., 1994). The authors described these cells as granulosa-luteal cells, although no *in vitro* differentiation process was described. Additionally, Peng et al. (1994) detected the presence of mRNA for GnRH itself in these cells. They found that GnRH regulated the expression of its receptor as well as its own message, with effects dependent on GnRH dosage.

It remains to be determined if mRNA for GnRH or the GnRH receptor can be detected in ovarian tissue of domestic animals. However, GnRH-like peptides have been found in bovine (Aten et al., 1987a; Ireland et al., 1988), ovine (Aten et al., 1987a) as well as rat (Aten et al., 1986) and human ovaries

(Aten et al., 1987b). These peptides appear to compete for GnRH binding sites but are not cross-reactive with GnRH antibodies. It is not clear whether these GnRH-like peptides have any real physiological role in regulating ovarian function.

The most likely route by which exogenous GnRH alters luteal function in domestic animals is indirectly via the endogenous release of LH. As discussed above, GnRH given at various times during the estrous cycle causes a rise in systemic LH. To examine how GnRH might act on the corpus luteum, Slayden and Stormshak (1990) on day 2 of the cycle injected GnRH directly into the artery of the ovary bearing the corpus luteum in ewes. In one experiment injection of 25 μg GnRH into the ovarian artery caused a reduction in serum concentrations of progesterone on days 7 through 11 of the cycle compared with saline-injected controls. This dosage, however, was actually high enough to cause a significant increase in systemic LH in response to the GnRH. When a second experiment was conducted using 50 ng of GnRH injected into the ovarian artery, no rise in LH and no change in serum concentrations of progesterone was found. Thus, it appears that GnRH most likely alters progesterone production by the corpus luteum *in vivo* by acting through an endogenous LH surge rather than directly on the ovary. This was further confirmed in a third experiment, when repeated injections of LH were given on day 2 via the jugular vein to mimic the serum profile of LH following a GnRH injection. In this experiment, serum progesterone concentrations were significantly depressed on days 6 and 8 of the estrous cycle (treatment × day interaction). Milvae et al. (1984) also attempted to examine local effects of GnRH on luteal function by using an intrauterine infusion of GnRH. Intrauterine infusion of 100 μg GnRH twice daily on days 12, 13 and 14 of the estrous cycle did increase jugular concentrations of LH, but plasma progesterone was not affected. The same study, however, did

show that a high dose of GnRH could directly depress progesterone production by isolated bovine luteal cells *in vitro* (Milvae et al., 1984). The decrease in progesterone was only detected at a high dose of GnRH (100 ng). The authors postulated that perhaps a non-receptor mediated mechanism was responsible for the inhibitory effect of the GnRH *in vitro*.

Practical Applications

The role of GnRH in altering ovarian or luteal function is of interest to animal producers, who could use it as a tool for enhancing reproductive efficiency (or who could avoid its use if it is detrimental to certain aspects of reproduction). Casida et al. (1944) first reported that sheep pituitary extract rich in LH could be used for treatment of ovarian cysts in cattle. Human chorionic gonadotropin, which possesses LH activity (Mason et al., 1962), was previously the most commonly used agent to treat ovarian cysts. Because injection of GnRH is known to cause a release of LH from the pituitary, GnRH was also found to rid animals of follicular cysts (Kittok et al., 1973; Bierschwal et al., 1975; Cantley et al., 1975; Seguin et al., 1976). Treatment with GnRH cures ovarian cysts in approximately 70 to 80% of cows, whether the cysts are of follicular or luteal origin. However, if the presence of a luteal cyst can be accurately determined, the use of $PGF_{2\alpha}$ or an analogue is recommended over the use of GnRH (Archbald et al., 1991).

Seguin et al. (1977) performed experiments to determine what effect hCG and GnRH would have on estrous cycle length in the event of a misdiagnosis of cystic ovaries (i.e., if the animal was actually in the luteal phase of the cycle). Repeated injections of hCG had been shown to be luteotropic and extend estrous

cycle length in cycling animals (Wiltbank et al., 1961; Moody et al., 1971; Veenhuizen et al., 1972), while a preliminary study (Britt, 1975) had shown that injection of GnRH into heifers on days 15, 17 or 19 did not affect estrous cycle duration, although behavioral estrus was inhibited in some of the animals injected on day 19. Seguin et al. (1977) injected heifers with saline, 100 μg GnRH or 10,000 U hCG on day 10 or 11 after estrus. In one experiment, they found that injection of hCG on day 10 or 15, but not day 17, prolonged luteal function and thus increased estrous cycle length by approximately 4 to 7 days. In a second experiment, administration of hCG on day 10 or 11 again extended the length of the estrous cycle, while GnRH did not alter estrous cycle length when compared with saline controls. Also, hCG, but not GnRH, increased serum progesterone between time of injection and estrus. Because the purpose of rupturing ovarian cysts is to allow the animal to return to estrus more quickly, they deemed the use of GnRH rather than hCG to be, generally, the better treatment for ovarian cysts, especially in the event of misdiagnosis. The only disadvantage of GnRH compared to hCG was that in previous studies GnRH injection during proestrus inhibited signs of behavioral estrus, although ovulation was not affected (Britt, 1975; Convey et al., 1976). Milvae et al. (1984) showed that repeated injections of GnRH analogue (but not native GnRH) during diestrus also increased cycle length, another example that type of injection and route or frequency of administration can contribute to differences in effects of the decapeptide.

Gonadotropin-releasing hormone has often been used at the time of artificial insemination in cattle. A number of studies have been conducted to examine how GnRH given at this time or at other times during the cycle affects conception or pregnancy rates as well as luteal function. Schels and Mostafawi (1978) claimed that GnRH injection into cows at the time of first postpartum insemination tended to increase the first service conception rate ($p<0.18$) as well

as the total pregnancy rate after three inseminations (saline control: 73.4% pregnant, GnRH: 81.6% pregnant; $p<0.2$). Injection of 5 μg of Buserelin, a potent GnRH analogue, was without effect on first insemination pregnancy rates when injected once between days 1 to 13 post-insemination (Macmillan et al., 1986). However 10 μg Buserelin increased pregnancy rates when given on days 11 to 13, but not days 7 to 10, post-insemination. Total pregnancy rates after the second insemination were also greater in animals that had received Buserelin during days 11 to 13 post-first-insemination. Stevenson et al. (1984) examined the effect of GnRH given at time of first, second or third service artificial insemination after $PGF_{2\alpha}$-induced luteal regression. They found that conception rate at second and third service tended to be higher in those animals injected with GnRH at the time of artificial insemination, with GnRH injection having no effect on first service conception rates (unlike the studies mentioned above) Serum concentrations of progesterone in the GnRH-treated animals were decreased during the first 21 days post-estrus. In another set of studies Mee et al. (1993) found that GnRH administration at time of artificial insemination increased pregnancy rates on days 42 to 56 post-insemination in cows eligible for third service. In this study, serum concentrations of progesterone were higher on days 4 to 8, and up to 40 days after treatment (during pregnancy) in cows injected with GnRH when compared with saline-injected controls.

Ellington et al. (1991) examined the effect of Buserelin injection on pregnancy rates in heifers used as embryo transfer recipients. Embryos were transferred on day 7 to 8 post-estrus and recipients received Buserelin injection at time of transfer or 4 to 7 days post-transfer. In this study no effect of Buserelin injection on pregnancy rates at days 35 to 60 post-insemination was detected.

Effects of GnRH on conception and embryo survival in other species has been examined as well. Injection of GnRH at time of mating of pubertal gilts

was found to increase number of ovulations, but was without effect on number of conceptuses and number of viable fetuses (Archibong et al., 1987). In ewes, preliminary studies indicated that injection of Buserelin 13 or 14 days after natural service increased lambing rate by 10% (unpublished data cited in Macmillan et al., 1986).

The mechanism by which GnRH might influence conception rates or embryonic survival is still unclear. Lucy and Stevenson (1986) suggested that the action of GnRH must be mediated by some other means than augmenting serum concentrations of progesterone in treated animals. This assumption seems to be correct, as various studies report both increases and decreases in serum progesterone after GnRH administration, while conception rates tend to be improved in either case.

STATEMENT OF THE PROBLEM

Embryonic mortality is of great concern to animal agriculture; it is estimated that 25 to 55% of all embryos die during early gestation (Niswender and Nett, 1994). Reproductive failure is considered to be one of the most costly and limiting factors in the livestock industry (Roberts et al., 1990). Some of these losses can be accounted for by genetic defects of the embryo itself, or environmental factors such as nutrition or climate, but maternal problems, especially of an endocrine nature, can be factors as well (Sreenan and Diskin, 1983). Animals known as "repeat breeders" who fail to conceive or maintain early pregnancy, and thus continue to cycle and are available for repeated breeding, certainly contribute to this loss.

Inadequate luteal function has been proposed as one mechanism by which animals fail to maintain early pregnancy. Kimura et al. (1987) suggested that delayed formation of the corpus luteum, either combined or not combined with lowered secretion of progesterone during the luteal phase, is one of the causes of repeat breeder syndrome. However, the primary function of the corpus luteum is to produce the steroid hormone progesterone, which prepares the uterus for implantation and helps maintain further luteal function and pregnancy. A need for adequate amounts of progesterone in early pregnancy is well established (Wilmut et al., 1985) and insufficient progesterone has been implicated in some studies as a factor in abnormal embryo development and early embryonic death (Sreenan and Diskin, 1983; Lamming et al., 1989). Thus the study of factors regulating corpus luteum function, including those affecting production of progesterone, are of importance for animal production.

Gonadotropin-releasing hormone has been used as one tool to combat economic losses due to repeat breeder syndrome and early embryonic death in

cattle (Schels and Mostafawi, 1978; Stevenson et al., 1984; Lucy and Stevenson, 1986; Macmillan et al., 1986 ; Mee et al., 1993), although its effects on serum concentrations of progesterone have been variable (Stevenson et al., 1984; Lucy and Stevenson, 1986; Rodger and Stormshak, 1986, Mee et al., 1993). Thus further study of how GnRH affects corpus luteum function is warranted. Mechanisms regulating luteolysis are also of interest, because if an animal fails to become pregnant, the corpus luteum needs to regress at the appropriate time in order to ensure a new cycle, and thus the potential for a new pregnancy. Early administration of GnRH seems to increase the large luteal cell to small luteal cell ratio in the corpus luteum (Mee et al., 1993). Products of the large luteal cell (e.g., oxytocin) are thought to be pivotal in the luteolytic process. Thus it is appropriate to examine whether GnRH given early in the estrous cycle can alter responses of the CL during luteal regression.

IN VITRO BOVINE LUTEAL PROGESTERONE PRODUCTION AFTER TREATMENT WITH GONADOTROPIN-RELEASING HORMONE IN VIVO

INTRODUCTION

Ford and Stormshak (1978) first observed that injection of gonadotropin-releasing hormone (GnRH) into cows during metestrus reduced serum concentrations of progesterone later in the estrous cycle. This effect was confirmed by Rodger and Stormshak (1986), who found that when GnRH was injected on day 2 of the estrous cycle, serum progesterone levels were lower than saline-treated controls beginning on day 8 of the cycle. The effect of GnRH on the corpus luteum appears to be indirect, at least in domestic animals. Brown and Reeves (1983) found no evidence for GnRH receptors on corpora lutea or follicles of cows, ewes or sows. They confirmed that the rat ovary does contain binding sites for GnRH. Additionally, Slayden and Stormshak (1990) found that, in ewes, injection of luteinizing hormone (LH) could mimic the effect of GnRH injection on serum progesterone levels later in the cycle, thus supporting the idea that the effects of GnRH injection in ruminants is due to a GnRH-induced LH surge.

The mechanisms of action of GnRH-induced alteration of luteal function are still unclear. Rodger and Stormshak (1986) found that GnRH injection on day 2 reduced number of luteal LH receptors on days 8 and 14 of the cycle and proposed this could directly be a reason for the altered luteal function, or that this could indicate more large luteal cells (and thus fewer LH receptors) were present in GnRH-exposed corpora lutea. Mee et al. (1993) confirmed this latter hypothesis; they found an increased large luteal cell to small luteal cell ratio in

CL of cows that had received GnRH 12 h after the onset of estrus. That study did not, however, rule out potential alteration in luteal function at the cellular level. In the present study, two experiments were conducted to further examine the action of exogenous GnRH on luteal cell function. More specifically, whether plasma membrane-related events contribute to altered luteal function, and whether large and(or) small luteal cells are functionally different in corpora lutea of animals exposed to GnRH compared with control animals.

MATERIALS AND METHODS

Experiment 1

Experiment 1 was conducted to determine if luteal cell membrane-related events contribute to attenuated progesterone production by corpora lutea of GnRH-treated heifers. Beef heifers of mixed breeds were checked twice daily for estrus using a vasectomized bull. On day 2 of the estrous cycle (estrus = day 0 of the cycle), animals were injected i.v. with 2 ml sterile saline (0.9% NaCl) or GnRH (Cystorelin®, 50 μg/ml, Sanofi Animal Health, Overland Park, KS; n = 5 animals per group).

On day 7, animals were restrained for surgery to remove the corpus luteum (CL). Caudal epidural anesthesia was induced by injection of 4 ml lidocaine hydrochloride (2%) into the coccygeal spinal column and an incision was made in the vagina through which the CL was removed. Corpora lutea were transported to the laboratory in sterile, phenol red-free Ham's F-12 medium [Nutrient Mixture F-12 (Ham), Gibco Laboratories, Inc., Grand Island, NY] containing 14 mM sodium bicarbonate, 24 mM HEPES and 30 μg/ml

gentamicin (Gibco), pH 7.3. For determination of adenylyl cyclase activity, approximately 100 mg of tissue were homogenized in 2 ml sucrose buffer [27% sucrose (w/w), 1 mM EDTA, 10 mM Tris, pH 7.5], immediately frozen in liquid nitrogen and stored at -80°C until the enzyme activity assay was performed.

The remainder of the CL was sliced to 0.3 mm thickness, washed three times in medium, and aliquoted to eight 10 ml Erlenmeyer flasks (approximately 100 mg per flask), each containing 2 ml incubation medium. Incubation medium consisted of Ham's F-12 (as described above) plus 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium (ITS, Sigma Chemical Co., St. Louis, MO). Treatments were then added to each flask: 20 μl saline were added to four flasks (two unincubated controls, two incubated controls), two flasks received bovine LH dissolved in 20 μl saline (final concentration: 50 ng/ml; USDA-bLH-B-6, USDA Animal Hormone Program, Beltsville, MD), and 8-bromo-cAMP (Sigma) was added to two flasks at a final concentration of 15 mM. Flasks were gassed with 95% O_2-5% CO_2 for several seconds each and capped with silicone stoppers. An additional 2 ml of cold ethanol was then immediately added to the two unincubated control flasks to preclude further progesterone synthesis. Two additional milliliters of ethanol were used to rinse the flask. The remaining flasks were incubated for 2 h at 37°C in a Dubnoff shaking water bath. After 2 h, cold ethanol was added to these flasks to terminate the incubation. Tissue plus medium samples were stored at -20°C until extraction and determination of progesterone content by radioimmunoassay.

Experiment 2

Experiment 2 was conducted to examine whether functional differences between large and(or) small luteal cells of saline vs. GnRH-treated heifers may contribute to altered progesterone production by GnRH-exposed corpora lutea. Beef heifers were checked twice daily for estrus using a vasectomized bull. On day 2 of the estrous cycle, 2 ml saline or 2 ml GnRH (100 μg) were injected i.v. as in Experiment 1 (n = 5 animals per treatment).

On day 7, a blood sample was taken for determination of serum concentration of progesterone and the CL removed via vaginal incision as described for Exp. 1. The CL was transported to the laboratory in Ham's F-12 where it was dissected free of connective tissue in a sterile cell culture hood. The tissue was then dissociated according to a modification of the procedure by Pate and Condon (1983). The CL was cubed into 1 mm pieces and minced finely with surgical scissors for 5 to 10 min in medium containing 0.5% bovine serum albumin (BSA, Sigma). Tissue was rinsed several times with medium and dissociated in a spinner flask for 1 h at 37°C in 20 to 25 ml 0.5% BSA medium containing 2000 U collagenase (CLS 1, 142 U/mg, Worthington Biochemical Corp., Freehold, NJ) per gram tissue. Tissue and medium were aspirated 5 to 10 times every 10 min during dissociation to aid in dispersion of cells. At the end of 1 h, cells were transferred to two 15 ml sterile centrifuge tubes and centrifuged at 150 × g for 15 min to remove collagenase. Supernatant was removed and cells resuspended in 0.5% BSA medium. Cells were washed again at 85 and 65 × g in a similar fashion. Cells were counted on a hemocytometer using the trypan blue dye exclusion method (Patterson, 1979) for determination of live and dead luteal cell numbers.

An aliquot (7.5×10^6 luteal cells) was removed and adjusted to a volume of 50 ml with 0.5% BSA medium for cell separation. The remaining cells were kept on ice in 0.5% BSA medium during the cell separation procedure. Cells were separated by sedimentation at unit gravity in an Eppendorf Celsep™ apparatus (Brinkmann Instruments, Inc., Westbury, NY) according to a modification of the procedures by Weber et al., (1987) and Dr. Gary Williams (Texas A&M University Agricultural Research Station, Beeville, TX, personal communication). A gradient of approximately 880 ml Ham's F-12 medium containing 1 to 2% BSA was created in the one liter separation chamber, with an underlay of 20% BSA medium. The cells in 0.5% BSA medium were loaded on top of the gradient; an overlay of 25 ml Ham's F-12 without BSA was loaded on top of the cells. Separation occurred for 1 h 15 min. During separation, the cell culture hood was shut off to avoid vibration. After separation, ten 15 ml and sixteen 50 ml fractions were collected into sterile centrifuge tubes. Cells were centrifuged at $150 \times g$ for 10 min and most of the medium was decanted. Dissociated cells that were previously held on ice were also centrifuged, then resuspended in Ham's F-12. Fractions from the cell separation were examined under a microscope using an ocular micrometer to determine cell types in each. Fractions containing small luteal cells (approximately 15 to 22 μm) with as few contaminating larger or smaller cells as possible were combined, an aliquot counted via hemocytometer, and then resuspended in Ham's F-12.

Both dissociated (mixed) and separated small luteal cells were plated onto one 6 well plate each (Corning Glass Works, Corning, NY). Cell culture plates used had earlier been serum-coated at 37°C for 1 to 4 h with Ham's F-12 containing 10% calf serum (Sigma), then rinsed 2 to 3 times with fresh medium before cells were added. Approximately 0.5 to 0.75×10^6 cells were added to each well in a final volume of 3 ml Ham's F-12 containing ITS. Cells were

incubated overnight at 37°C in a humidified incubator with a 5% CO_2 atmosphere. The following day, the medium was removed from all wells and 3 ml fresh medium with ITS were added. Treatments were added in duplicate to wells containing small and mixed cells. Treatments consisted of: control (medium alone), LH (50 ng/ml) and 8-bromo-cAMP (15 mM). Cells were incubated for 2 h; the media from each well were then recovered and stored frozen at -20°C until analysis for progesterone. Additional Ham's F-12 was added to each well and cells were counted using a Nikon TMS inverted phase light microscope at 200X magnification. Large and small cells were distinguished morphologically.

Progesterone Radioimmunoassay

Tissue plus medium from Exp. 1 were extracted by modification of the procedure described by Koligian and Stormshak (1976). Each sample (tissue plus medium) was placed into a Duall ground glass homogenizer after addition of 20,000 cpm of [^{3}H]progesterone (Dupont NEN®, Boston, MA) to the sample storage vial. The sample storage vial was rinsed three times with 2 ml ethanol and the sample homogenized. The homogenate was poured over Whatman No. 1 filter paper, which was held in a glass funnel, and allowed to filter through into round bottom flasks. The glass homogenizer and pestle were rinsed four times with 2 ml ethanol and these rinses were added to the filter paper. The filter paper was rinsed five times with 2 ml ethanol and allowed to dry; the filter was then removed and the glass funnel and inner neck of the flask were rinsed with ethanol. Flasks were roto-evaporated at 45°C until samples were nearly dry, then 3 ml distilled water were added and the samples vortexed for 30 sec.

Samples stood at room temperature for 30 min and were then vortexed for 2 min after the addition of 20 ml benzene:hexane (1:2). After storage at -20°C overnight, the organic phase was decanted and dried under air. Samples were resuspended in 20 ml ethanol and a 1 ml aliquot was removed to determine extraction efficiency. Sera from Exp. 2 were also extracted similarly by vortexing for 30 sec with 2 ml benzene:hexane (1:2) and dried down for use in the assay, while media progesterone concentrations (Exp. 2) were determined by radioimmunoassay without extraction. The mean extraction efficiency for tissue plus medium samples from Exp. 1 was 68.7±0.8%. Each tissue plus medium sample was corrected using its specific extraction efficiency. Mean extraction efficiency for serum from Exp. 2 was 88.7%, which was used as a correction factor for all samples.

Radioimmunoassay was performed using progesterone standards ranging from 5 pg/100 µl to 800 pg/100µl in ethanol. Ethanol standards (100 µl, in triplicate) or diluted sample (100 µl, in duplicate) were pipetted into 12 × 75 mm glass test tubes and evaporated. One hundred microliters of phosphate buffered saline with gelatin (GPBS; 0.01 M $NaPO_4$ pH 7.0, 0.14 M NaCl, 1:10,000 thimerosol, 0.1% gelatin) was then added to all tubes. Aqueous (media) samples (100 µl) were not evaporated; an additional 100 µl of GPBS were added to all other tubes to compensate for the additional volume. Anti-progesterone-11-BSA (Dr. Gordon Niswender, Colorado State University, Fort Collins, CO) in 100 µl GPBS was added to each standard and sample tube in a 1:3500 dilution. Tubes were incubated 30 min at room temperature and 20,000 cpm [^{3}H]progesterone in 100 µl GPBS were then added to each tube. Tubes were vortexed then incubated overnight at 4°C. The following day, 1 ml of dextran-coated charcoal [2.5 g/l washed neutral norit charcoal (Sigma), 0.25 g/l Dextran T-70 (Pharmacia, Uppsala, Sweden)] in GPBS was added rapidly to each tubes. Tubes were

vortexed and incubated at 4°C for 15 min, then centrifuged at 2540 × g for 10 min. The supernatant from each tube was poured into a 20 ml glass scintillation vial and 6 ml of Ecolume scintillation cocktail (ICN, Costa Mesa, CA) were added. Samples were counted in a Beckman LS-6000 liquid scintillation counter. Standard curves were plotted and unknown concentrations determined using the RIA AID computer program (Robert Maciel Associates, Inc., Arlington, MA). Intraassay and interassay coefficients of variation were determined using a 75 pg/100 µl sample in ethanol (18 tubes per assay), and for Exp 1. were 10.1±0.5% and 3.8% (n = 7 assays), and for Exp. 2 were 10.9±0.7% and 6.8% (n = 8 assays), respectively.

Adenylyl Cyclase Activity Assay

Adenylyl cyclase activity was measured in luteal homogenates by modification of procedures by Agudo et al. (1984) and Birnbaumer et al. (1988). All chemicals were purchased from Sigma Chemical Co. unless otherwise noted. Each sample (50 µl) was incubated in the presence of 10 µl 10X incubation mixture, pH 7.3 [0.5 mM HEPES, 50 mM $MgSO_4$, 20 mg/ml BSA, 10 mM cAMP and 1×10^7 cpm [^{3}H]cAMP (Dupont NEN)], 10 µl 10X regeneration mixture [40 mM creatine phosphate, 25 units creatine phosphokinase (Calbiochem, San Diego, CA)] and 20 µl 5X ATP [0.5 mM containing approximately 1×10^6 cpm [α-^{32}P]ATP (Dupont NEN)] in a total volume of 100 µl . Actual specific activity of the 5X ATP mixture was determined by use of a spectrophotometer (ATP concentration) and the liquid scintillation counter (radioactivity). Adenylyl cyclase activity was determined in both the presence (activated) and absence (non-activated) of 0.1 mM GTPγS, each in duplicate for each luteal sample. A

reaction mixture without luteal homogenate was incubated with each set of samples as a reaction blank to determine non-specific incorporation. Reaction mixtures were incubated for 10 min at 30°C and the reaction stopped with 1 ml 0.17 N perchloric acid.

Stopped reaction mixtures were transferred onto individual Econo-pak glass columns 0.5 cm I.D., 15 cm long (Bio-Rad Laboratories, Hercules, CA) containing AG 50W-4X, 200-400 mesh, H^+ form resin (Bio-Rad) which had been subjected to three regeneration cycles (one cycle = 20 ml H_2O, 20 ml 2 N NaOH, 20 ml H_20, 20 ml 2 N HCl then 60 ml H_2O) before use. After the reaction mixture filtered into the resin bed, 1 ml then 2.5 ml H_2O were added to the top of the column. Each column was then placed above another glass column containing 1.2 g alumina oxide which had been rinsed with 10 ml 0.1 M imidazole-HCl, pH 7.5, before use. Samples were eluted from the resin column with 4 ml H_2O and allowed to drip into the alumina oxide column. Once the water had passed through the alumina oxide column, 1 ml of imidazole buffer was added to each column. Cyclic AMP was eluted into 20 ml scintillation vials by addition of 4 ml imidazole buffer. Fifteen milliliters of Ecolume were added to each vial and samples were counted using the liquid scintillation counter. [^{3}H]cAMP in eluted samples and in the original 10X incubation mixture were used to determine percentage recovery. [^{32}P]cAMP in the samples was used to determine conversion from [^{32}P]ATP with [^{32}P] in the reaction blank subtracted from each sample. Protein concentration in each luteal homogenate was determined using the Bio-Rad protein assay and adenylyl cyclase activity was expressed as cAMP formed in pmol•min^{-1}•mg protein^{-1}.

Statistical Analysis

Progesterone data for both experiments were analyzed by ANOVA using the general linear model procedures of SAS (1993). Treatment duplicates were averaged and the mean values used for statistical analysis. In Exp. 1, progesterone data were analyzed as a split-plot design, after subtraction of each sample's corresponding unincubated control, with GnRH, animal(GnRH), *in vitro* treatment and GnRH × *in vitro* treatment included as sources of variation. Animal(GnRH) was used as the error term to test the significance of GnRH injection. Only the GnRH effect is presented in the results because there were no GnRH × *in vitro* treatment interactions. Differences among specific treatment means were determined by Least Significant Difference test. Differences between mean non-activated or activated adenylyl cyclase activities were determined by t-test using Statgraphics (STSC, Inc., Rockville, MD).

For Exp. 2, progesterone data were analyzed as a split-split-plot using GnRH, animal(GnRH), cell type, GnRH × cell type, cell type × animal(GnRH), *in vitro* treatment, GnRH × *in vitro* treatment, cell type × *in vitro* treatment and GnRH × cell type × *in vitro* treatment as sources of variation. GnRH was tested for significance using animal(GnRH) as the error term; cell type and GnRH × cell type were tested against cell type × animal(GnRH) as the error term. Differences among treatment group means were determined using the contrast procedure of SAS. The percentages of large cells present in the mixed cell cultures were analyzed independently as a split-plot design with SAS, using GnRH, animal(GnRH), *in vitro* treatment and GnRH × treatment as sources of variation. GnRH was tested for significance using animal(GnRH) as the error term. Serum concentrations of progesterone for Exp. 2 were analyzed by t-test with Statgraphics. Statistical consultation for Exp. 1 was provided by Dr. Ken Rowe,

and for Exp. 2 by Dr. David Thomas, both of the Department of Statistics, Oregon State University.

RESULTS

In Exp. 1, GnRH injection on day 2 significantly reduced progesterone production by luteal slices on day 7 of the cycle in response to LH ($p<0.01$) and cAMP ($p<0.001$) but did not affect basal progesterone production by the tissue slices ($p>0.05$; Figure 1). There were overall effects of GnRH to decrease progesterone production ($p<0.05$) and of the LH and cAMP *in vitro* treatments to stimulate progesterone production ($p=0.0001$). Neither non-activated adenylyl cyclase activity nor that activated with GTPγS was affected by *in vivo* injection with GnRH ($p>0.05$; Figure 2).

In contrast, progesterone production in Exp. 2 by small or mixed luteal cells in response to LH or cAMP was not affected by *in vivo* administration of GnRH ($p>0.05$; Figure 3A). There was an overall effect of *in vitro* treatment ($p=0.001$); LH and cAMP stimulated progesterone production compared to *in vitro* controls (saline: small cells $p<0.002$, mixed cells $p=0.001$; GnRH: small cells $p<0.001$, mixed cells $p<0.001$). Also, mixed cell cultures had higher progesterone production than small cell cultures ($p<0.001$), although small cells appeared to have greater response to the *in vitro* treatments when data were expressed as a percentage of the respective *in vitro* controls (Figure 3B). When numbers of large cells present in the mixed cell cultures (expressed as a percentage of total cells) were analyzed, there were no differences between GnRH and saline injections (saline: 3.61±0.35%; GnRH: 3.65±0.28%; $p=0.95$). Also, GnRH injection on day 2

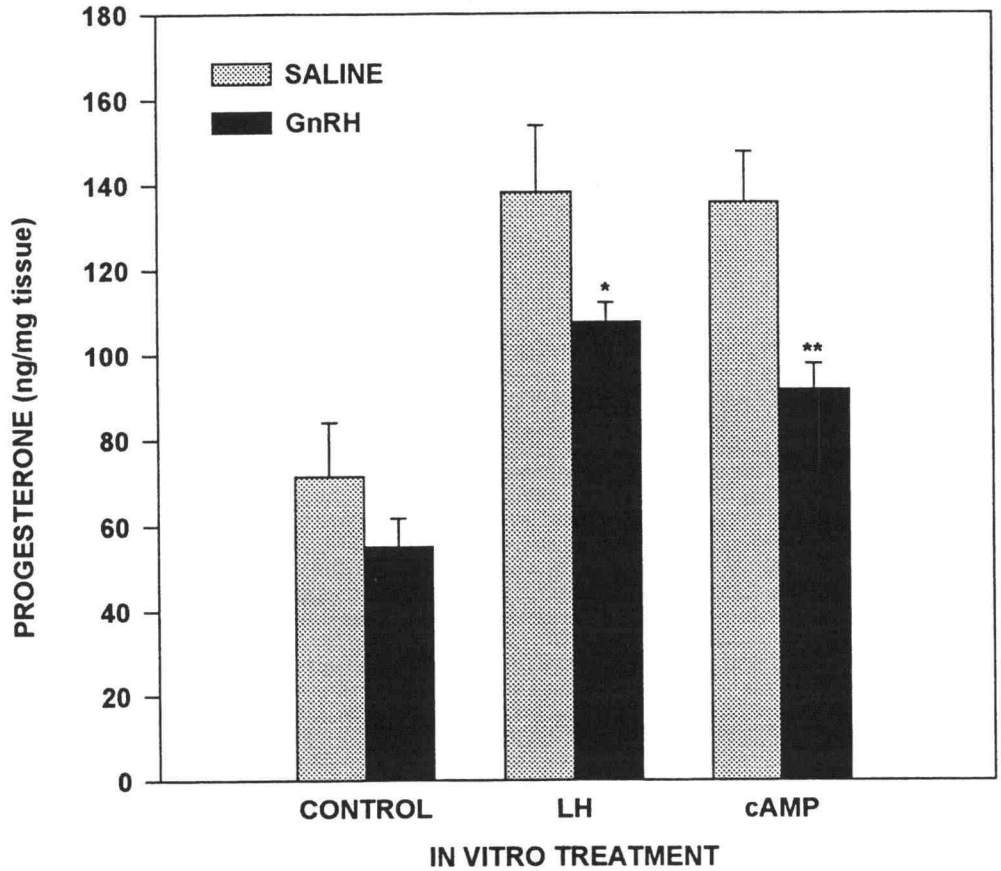

Figure 1. *In vitro* progesterone production by day 7 luteal slices from beef heifers injected with saline or GnRH on day 2 of the estrous cycle. Treatments imposed *in vitro* were LH (50 ng/ml) and 8-bromo-cAMP (15 mM). The overall effects of GnRH injection ($p<0.05$) and *in vitro* treatments ($p=0.001$) were significant. *In vivo* administration of GnRH reduced progesterone production by LH (*, $p<0.01$) and cAMP (**, $p<0.001$) treated luteal slices only.

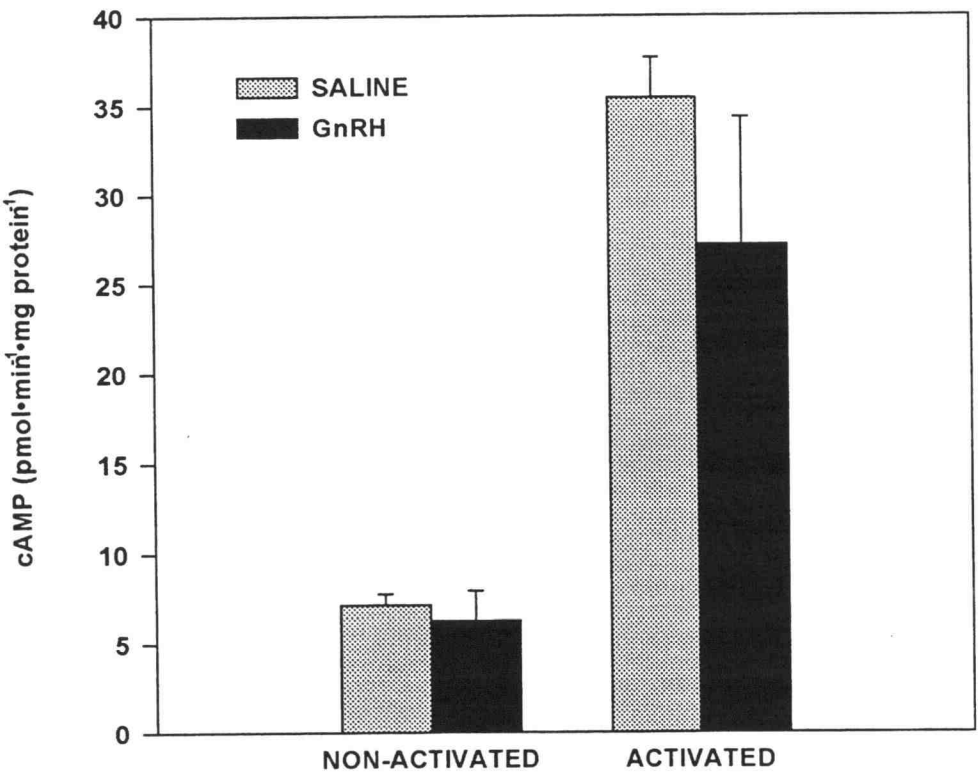

Figure 2. Adenylyl cyclase activity in corpora lutea of beef heifers injected with saline or GnRH expressed as pmol cAMP formed•min^{-1}•mg protein^{-1}. The enzyme assay was performed without (non-activated) or with (activated) the addition of 0.1 mM GTPγS. Administration of GnRH was without effect on adenylyl cyclase activity ($p>0.05$).

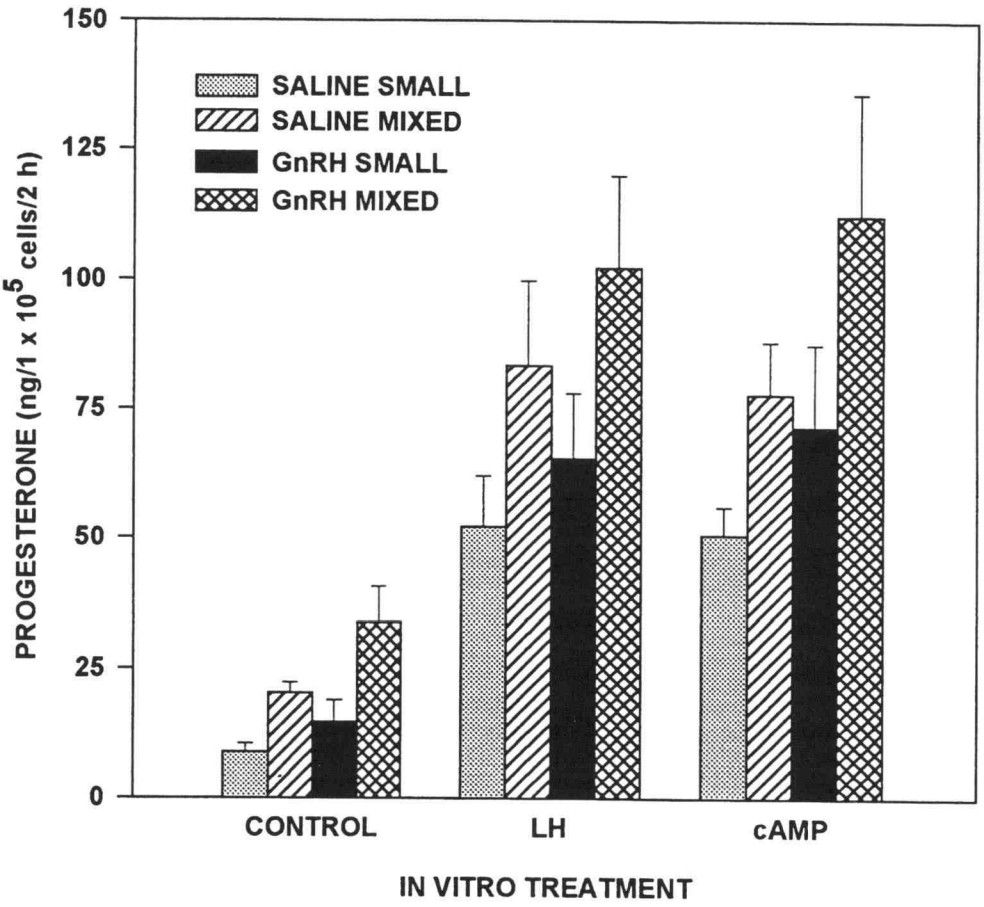

Figure 3A. Progesterone production by small and mixed luteal cell cultures from corpora lutea of beef heifers injected early in the estrous cycle with saline or GnRH. *In vitro* treatment with LH or cAMP significantly stimulated progesterone production compared with controls ($p<0.005$); however, *in vivo* administration of GnRH had no effect on progesterone production.

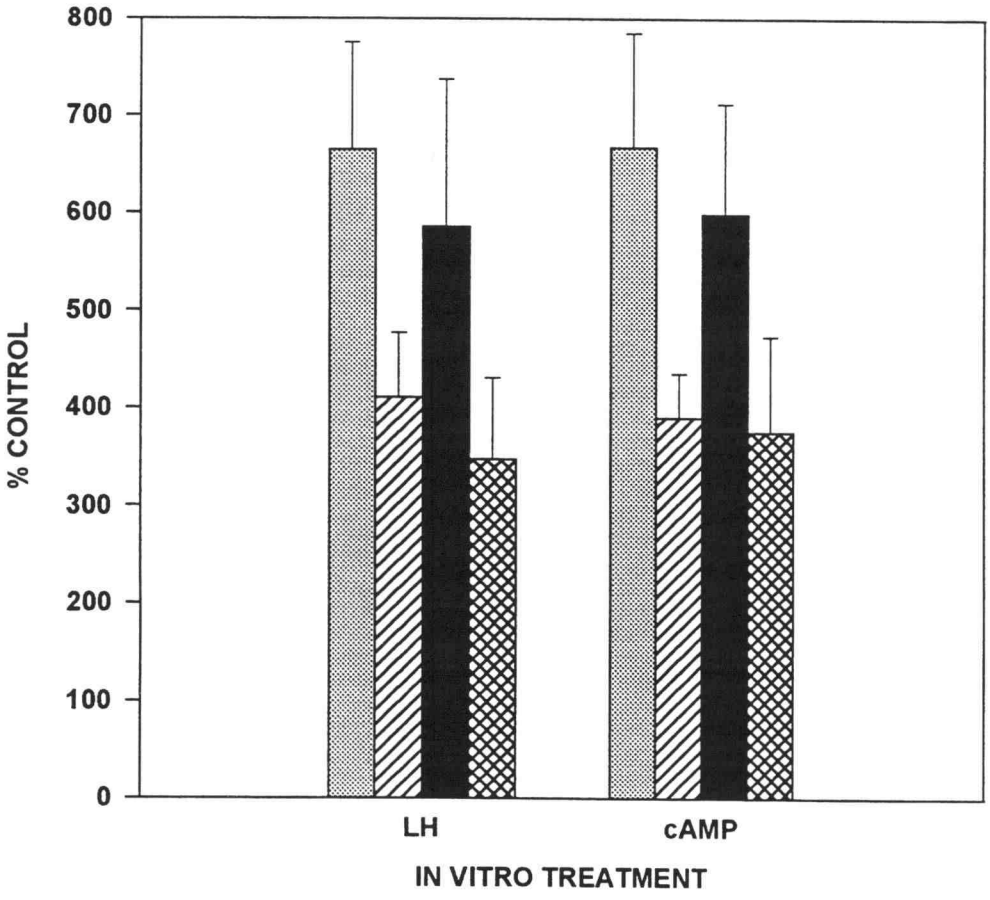

Figure 3B. Data from Fig. 3A presented as a percentage of *in vitro* control treatment.

of the cycle did not affect serum progesterone on day 7 in the present study (saline: 2.55±0.48 ng/ml; GnRH: 2.84±0.21 ng/ml; $p>0.05$).

DISCUSSION

Results from Exp. 1 suggest two potential mechanisms that may explain the alteration in luteal function found after GnRH injection. One possibility is that cellular components responsible for progesterone production by normal LH stimulation are functionally altered. This could explain why progesterone production was reduced in GnRH-exposed luteal tissue in response to both LH and cAMP (Fig. 1). If this is true, then this functional difference must occur at a point distal to the accumulation of cAMP in the luteal cell. In this experiment, stimulation of progesterone with cAMP was depressed in corpora lutea from GnRH-treated heifers compared with controls. In addition, the response of this tissue to LH was similar to the response to cAMP. The finding that there is no difference in luteal adenylyl cyclase activity between saline- and GnRH-treated animals (Fig. 2) further supports this premise, because adenylyl cyclase is the enzyme responsible for the conversion of ATP to cAMP. Thus the adenylyl cyclase/cAMP system was not able to overcome the GnRH-induced inhibition of progesterone production by corpora lutea in response to LH *in vitro*.

This idea is consistent with results from Rusbridge et al. (1993) who performed an experiment using dissociated luteal cells from untreated heifers on day 7 of the cycle, or heifers injected with GnRH on day 6 followed by CL removal on day 13 of the estrous cycle. Their results of stimulation of the cells with LH and dibutyryl cAMP led them to conclude that alteration in steroidogenic response of GnRH-exposed corpora lutea occurs at least at a point

distal to the LH receptor. There are a number of other steps in the biochemical pathway leading to production of progesterone by the luteal cell that could potentially be affected subsequent to GnRH injection early in the cycle. These include the cytoplasmic enzyme protein kinase A (PKA), also known as cAMP-dependent protein kinase; the transport and mobilization of the steroid hormone precursor, cholesterol, into the mitochondria; the action of cytochrome P450 side chain cleavage complex, the enzyme system responsible for the conversion of cholesterol to pregnenolone; or the enzyme activity of 3β-hydroxysteroid dehydrogenase, which converts pregnenolone to progesterone (Leers-Sucheta and Stormshak, 1991).

A second possibility is that no functional difference exists between corpora lutea of animals that have received an injection of GnRH and controls. Change in cellular composition of the corpus luteum has been proposed as the mechanism by which alteration of luteal function occurs. Mee et al. (1993) found that GnRH administered 12 h after the onset of $PGF_{2\alpha}$-induced estrus (at the time of artificial insemination) in repeat-breeder dairy cows increased the large to small luteal cell ratio in corpora lutea from these animals on day 10 after estrus. Saline-injected control animals had a ratio of 14% large to 86% small cells, while those cows that received GnRH had a ratio of 31% large to 69% small cells. These researchers conducted an *in vitro* experiment as well, and found that early administration of GnRH did not affect basal progesterone production by day 10 luteal tissue slices but reduced LH-stimulated progesterone production. These data are similar to those in Exp. 1 of our study. *In vivo* experiments from Mee et al. (1993) indicated that serum concentrations of progesterone tended to be greater in both pregnant and nonpregnant GnRH-injected animals compared with similar saline controls. This is somewhat surprising, because in an earlier report (Lucy and Stevenson, 1986), the same laboratory reported a decrease in

serum concentrations of progesterone in response to early GnRH administration under similar experimental conditions. Both studies reported an increase in pregnancy rates in the GnRH-injected animals compared with saline-injected controls. Lucy and Stevenson (1986) explained that although serum progesterone was lower in GnRH-treated cows, a slower rise in progesterone may have aided in embryo survival. Mee et al. (1993) reported an earlier rise in progesterone in GnRH-treated animals, and stated that pregnancy rates were better in animals that had received an injection of GnRH because of greater serum concentrations of progesterone (due to high basal progesterone production from the CL). They attributed the latter to the increased number of large luteal cells. Large luteal cells contain few LH receptors and produce high basal amounts of progesterone, while small luteal cells have many LH receptors and produce increased quantities of progesterone in response to LH (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Fitz et al., 1982).

Their findings, however, cannot rule out the possibility of further differences in function of the luteal cells between saline- and GnRH-treated animals. It seems likely that if an increased large luteal cell to small luteal cell ratio is the only explanation for the findings by Mee et al. (1993) and the results of Exp. 1, then significantly higher basal progesterone production by GnRH-exposed corpora lutea should occur, rather than just a reduction in LH (and cAMP)-stimulated progesterone production. However, this was not the case in either study. Thus it was anticipated that results of Exp. 2 would further define differences present in large and(or) small luteal cells from GnRH-exposed animals.

Results from Exp. 2 indicate that, in the cell culture system utilized, both isolated small luteal cells and mixed luteal cells were able to produce the same quantity of progesterone basally and in response to stimulation by LH and

cAMP whether they were derived from saline or GnRH-treated animals (Figs. 3A and 3B). These data suggest that no functional difference exists between luteal cells of GnRH- or saline-injected animals, and that a difference in proportion of large luteal cells and small luteal cells may indeed account for alterations in luteal hormone production. The similarity in percentages of large luteal cells found in mixed luteal cell cultures between saline and GnRH-treated animals may be explained by the fragility of the large luteal cells of the bovine during the dissociation procedure. It is known that selective losses of large luteal cells occur during cell dispersion and purification procedures (Ursely and Leymarie, 1979; Chegini et al., 1984; Hansel et al., 1987; O'Shea et al., 1989; Lei et al., 1991). It is possible that, if GnRH-exposed corpora lutea had an increased number of large luteal cells, that these could be even more delicate than those normally found, such as those of control corpora lutea. Thus a selective loss of large luteal cells between the two treatments might have normalized the large cell numbers found in the mixed cell cultures of the GnRH- and saline-exposed corpora lutea.

Other factors could also provide explanation for the results found. For example, the potential importance of contact-dependent intercellular communication cannot be discounted as a reason for the different results found in Exps. 1 and 2. It is known that gap junctions, junctions of communication or electrical coupling, exist in the corpus luteum of a number of species including mice, rat, rabbit, dog, monkey and human (Enders, 1973; Abel et al., 1975a, 1975b; Albertini and Anderson, 1975; Gulyas et al., 1976; Crisp and Dessouky, 1980). The existence of junctional structures among contacting ovine luteal cells has also been reported (McClellan et al., 1975; O'Shea et al., 1979). Redmer et al. (1991) found that mid-cycle bovine luteal cells preincubated for 24 to 48 h then cultured for 16 to 24 h with LH, $PGF_{2\alpha}$ or forskolin (a compound that directly

activates adenylyl cyclase) had significantly higher rates of large to small cell communication than those incubated with no hormone. Additionally, forskolin increased communication between small cells. Thus, bovine luteal cells are capable of intercellular communication and the rate of communication can be influenced by hormones, implying a potential role for contact-dependent communication in regulation of luteal function. In the present study, tissue used in Exp. 1 would certainly maintain any gap junctions that would normally exist between luteal cells. In Exp. 2 these junctions would generally be disrupted during the dissociation process but could be reestablished during cell culture. However, the cells in Exp. 2 were maintained in culture for a shorter period of time than those in the study by Redmer et al. (1991). For example, in Exp. 2 an overnight preincubation step (approximately 16 to 18 h) occurred before measurement of hormone production, rather than 24 to 48 h, as in the study by Redmer et al. (1991). This additional time may be important for establishment of cell-to-cell contacts. Also, as stated above, communication between cells was greater in those cultures exposed to hormones for the 16 to 24 h period. Thus it appears that hormonal stimulation is important for formation of cellular contacts. In Exp. 2, cells were incubated with hormone for 2 h, a period of time that certainly allows stimulation of progesterone production by the cells but which may not be long enough to allow for the promotion of intercellular contacts.

The contribution of other cell types may also be important. Endothelial cells, which are present in large numbers in the highly vascularized corpus luteum, have recently been shown to interact with luteal cells (Girsh et al., 1995). According to the description of large, small and endothelial cell cultures by Girsh et al. (1995), it is likely that the cell cultures in Exp. 2 did contain endothelial cells. Endothelial cells were certainly present in the intact luteal

slices from Exp. 1. Again, cell-to-cell contact may be important, as it seems that endothelial cells can form contacts with both small and large luteal-like cells in culture and this could be one way by which these vascular cells affect luteal function. However, they are also known to secrete PGI_2, and this may be in important mediator in regulating luteal function.

In summary, early administration of GnRH inhibited LH and cAMP-stimulated progesterone production from day 7 bovine luteal slices but not from day 7 luteal cells after overnight cell culture. The results may be explained in a manner consistent with the hypothesis that an increased large luteal cell to small luteal cell ratio exists after GnRH injection, and that any effects of GnRH treatment can be attributed to this phenomenon. Still, it is difficult to explain, if an increased large luteal cell to small luteal cell ratio is the sole cause of altered luteal function in response to GnRH administration, why basal progesterone production by luteal slices did not increase (Exp. 1 and Mee et al., 1993). The possibility of functional differences in cells (luteal or nonluteal) of intact corpora lutea cannot be fully excluded.

LUTEOLYTIC RESPONSES OF THE BOVINE CORPUS LUTEUM AFTER EXPOSURE TO EXOGENOUS GONADOTROPIN-RELEASING HORMONE

INTRODUCTION

Few studies have examined responses of the corpus luteum to injection of $PGF_{2\alpha}$ after previous administration of GnRH. Injection of GnRH analogue into heifers and cows, followed by $PGF_{2\alpha}$ 6 or 7 days later, improved precision of estrous synchronization (Thatcher et al., 1989b; Twagiramungu et al., 1992) and allowed for increased pregnancy rates (Twagiramungu et al., 1992). This injection regimen was proposed as an alternate method of estrous synchronization that allowed for decreased estrous detection. Macmillan et al. (1985) reported that administration of GnRH at various times during diestrus, followed by injection of $PGF_{2\alpha}$ 15 min, 24 h or 72 h later did not affect decline in plasma progesterone caused by $PGF_{2\alpha}$ but did seem to inhibit structural demise of the CL. However, studies specifically examining the effect of GnRH injection early in the estrous cycle on $PGF_{2\alpha}$-induced luteal regression in mid-cycle have not been conducted.

Oxytocin is a hormone produced by large luteal cells , stored in secretory granules and secreted by exocytosis through the activation of PKC (reviewed by Stormshak et al., 1995). Release of oxytocin, through a protein kinase C-dependent mechanism in response to $PGF_{2\alpha}$, is an indicator that the CL is undergoing luteolysis. Because there is thought to be an increased large luteal cell to small luteal cell ratio in corpora lutea exposed early in the estrous cycle to GnRH (Mee et al., 1993), it is possible that GnRH-exposed corpora lutea would respond to the luteolytic stimulus $PGF_{2\alpha}$ with an increased secretion of oxytocin.

Davis et al. (1995) have shown that $PGF_{2\alpha}$-stimulated PKC activates the MAP kinase cascade in causing the release of TGFβ from bovine luteal cells. They hypothesized that this cascade causes phosphorylation of transcription factors in the luteal cell, presumably including c-jun (Khan et al., 1993, 1994). Activated transcription factors can then bind regulatory sites such as the AP-1 site on genes such as TGFβ to up-regulate expression and subsequent secretion. Thus, it is possible that other $PGF_{2\alpha}$-mediated exocytotic events from luteal cells can occur in a similar manner. Additionally, it has been suggested that the early response gene c-jun and its product may play a role in luteolysis (Khan 1993, 1994). Therefore the objective of the present study was to determine if the $PGF_{2\alpha}$-induced up-regulation of c-jun message in the bovine CL could be altered by early administration of GnRH. A corollary objective was to determine if GnRH administration can alter $PGF_{2\alpha}$-induced oxytocin secretion on day 8 of the estrous cycle.

MATERIALS AND METHODS

Experimental Design

The present experiment was conducted to determine whether exogenous GnRH can alter responses of the corpus luteum to exogenous prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). A preliminary experiment was first performed to determine if $PGF_{2\alpha}$ can alter c-jun expression in the bovine corpus luteum as has been described for the rat (Khan et al., 1993, 1994). Two beef heifers were injected with 500 μg cloprostenol, a $PGF_{2\alpha}$ analogue, (Estrumate, Mobay Corp., Shawnee, KS) i.v. on day 8 of the estrous cycle; one heifer received an injection of saline. Corpora

lutea were removed 60 min after injection, frozen in liquid nitrogen and stored at -80°C until RNA analysis. An additional day 8 corpus luteum from a heifer that did not receive an injection also served as a control.

For the actual experiment, beef heifers were injected with saline or GnRH on day 2 of the estrous cycle (n = 4 animals per group). On day 8 of the cycle, animals were restrained and the jugular vein catheterized with a 16-gauge, 8.3 cm Angiocath® catheter (Deseret Medical Inc., Becton Dickinson and Co., Sandy, UT). Clotting in the catheter was prevented by infusion of a 3.5% sodium citrate-0.2% oxytetracycline solution. Blood samples were then collected with Vacutainer tubes (Becton Dickinson and Co., Rutherford, NJ) for determination of serum progesterone or plasma oxytocin (collected in heparinized Vacutainers) and designated "time zero" samples. Ten microliters of 5 mg/ml 1,10-phenanthroline (Sigma) and 20 µl of 0.5 M EDTA were added immediately to this and all subsequent oxytocin samples to prevent oxytocinase activity (Kumarasen et al., 1974). All blood samples were placed on ice after collection. Next 500 µg cloprostenol ($PGF_{2\alpha}$)was injected via the catheter (time zero). Blood samples for oxytocin were collected as above at 1.5, 3, 6, 9, 12, 15, 20, 25, 30 and 35 min after $PGF_{2\alpha}$ injection. Caudal epidural anesthesia was then induced with 2% lidocaine hydrochloride and a vaginal incision was made for removal of the corpus luteum. At 60 min after injection, a blood sample was taken for determination of progesterone and the CL was removed and frozen in liquid nitrogen. Corpora lutea were stored at -80°C until analysis for c-jun expression and tissue concentrations of oxytocin. Blood samples were centrifuged for 10 to 15 min at 2540 × g at 4°C. Serum samples were allowed to clot at 4°C overnight before centrifugation. Both plasma and serum were stored at -20°C until hormone radioimmunoassay.

Oxytocin Radioimmunoassay

Oxytocin in plasma samples was extracted and assayed as recently described by Orwig et al. (1994) by methods adapted from Abdelgadir et al. (1987) and Schams (1983). Oxytocin was extracted from tissue samples by the method of Tsang et al. (1990) before analysis. For the plasma extraction, a Waters vacuum manifold was utilized with Sep-Pak® Plus C-18 cartridges (Waters Chromatography Division, Millipore Corp., Milford, MA). Mean extraction efficiency for plasma was 75.0±0.7% and for tissue 93.6±1.6%. Oxytocin antibody was generously provided by Dr. Dieter Schams, Technical University of Munich, Freising-Weihenstephan, Germany. Intra- and interassay coefficients of variation were 9.6±0.7% and 3.4%, respectively.

Progesterone Radioimmunoassay

Progesterone in duplicate serum samples (100 µl each) was extracted with 2 ml benzene:hexane (1:2) by vortexing for 30 sec. Samples were placed at -20°C overnight and the next day the organic phase was decanted into new tubes, dried down and used in the assay. A third tube for each sample containing 100 µl serum plus 4800 cpm [^{3}H]progesterone was extracted for determination of extraction efficiency. Mean extraction efficiency, used for all samples, was 85.5%. Radioimmunoassay was performed using progesterone standards (5 pg/tube to 800 pg/tube), anti-progesterone-11-BSA (Dr. Gordon Niswender, Colorado State University, Fort Collins, CO) and 20,000 cpm/tube of [^{3}H]progesterone (Dupont NEN). Bound and free fractions were separated

using dextran-coated charcoal. The intrassay coefficient of variation was 7.34% (n = 1 assay).

RNA Extraction and Northern Blotting

For extraction of RNA, luteal tissue (approximately 300 mg) was pulverized with a mortar and pestle under liquid nitrogen then placed in 10 volumes (3 ml) TRIzol® reagent (Gibco), a monophasic solution of phenol and guanidine isothiocyanate (Chomczynski, 1993). Extraction was performed according to the manufacturer's protocol, which included addition of chloroform, an isopropanol precipitation and centrifugation steps. Recovered RNA was dissolved in 50 µl diethylpyrocarbonate (DEPC) treated water. Quantity and purity of RNA was determined spectrophotometrically.

To denature the RNA, samples were heated to 55°C for 15 min with sample-preparation buffer in a ratio of 9:31. Sample-preparation buffer consisted of 1 part 10X MOPS buffer (0.2 M MOPS, 80 mM sodium acetate, pH 7.0), 1.75 parts deionized formaldehyde and 5 parts deionized formamide. Denatured samples plus 2 µl gel-loading buffer (50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were loaded onto a formaldehyde agarose gel (1%) containing approximately 0.8 µg/ml ethidium bromide. Electrophoresis was performed overnight at 30 V in 1X MOPS buffer.

After electrophoresis, the gel was rinsed for 1 h in DEPC-treated water, RNA bands were visualized under UV light and photographed. The gel was then soaked in 6X SSC buffer for 10 to 15 min (1X SSC = 0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0). RNA was transferred onto a Nytran Plus nylon membrane (Schleicher & Schuell, Keene, NH) by capillary transfer. After 48 h,

the membrane was rinsed briefly with 5X SSC then UV cross-linked at 120,000 $\mu J/cm^2$.

The membrane was prehybridized at 42°C for at least 6 h with 200 $\mu l/cm^2$ prehybridization solution [50% deionized formamide, 5X SSC, 50 mM K_2PO_4 pH 8.0, 5X Denhart's solution (0.1% each BSA, Ficoll and polyvinylpyrrolidone; Sigma), 100 μg/ml salmon testes DNA for hybridization (Sigma) and 0.1% SDS] in a rotating hybridization oven (Robbins Scientific, Sunnyvale, CA). The membrane was then hybridized with the appropriate labeled cDNA probe. The probe for c-jun was made from a plasmid insert of the full-length mouse c-jun cDNA obtained from Dr. Rodrigo Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ. Probes made from this template have been successfully used to examine c-jun expression in bovine tissue (Clark et al., 1992). The probe for 18S ribosomal RNA was made from a plasmid insert containing 80 bp of the human 18S rRNA gene (Ambion, Austin, TX). This sequence is highly conserved and can be used as a template to make an 18S probe that will bind to this rRNA from all vertebrates with few, if any, mismatches. Probes were made using the appropriate cDNA template by random hexanucleotide priming with [^{32}P]dCTP (Dupont NEN) as the radioactive label (Prime-a-Gene® Labelling System, Promega, Madison, WI). Unincorporated label was removed by passage though a Sephadex® G-50 column (Quick Spin™ columns, Boehringer Mannheim, Indianapolis, IN).

Labeled probe was added to the hybridization solution to obtain approximately 1.5 to 3 × 10^6 cpm/ml. Hybridization solution (50 $\mu l/cm^2$ membrane) contained 50% deionized formamide, 5X SSC, 20 mM K_2PO_4 pH 6.5, 1X Denhart's solution, 100 μg/ml salmon testes DNA for hybridization and 0.1% SDS. After the overnight hybridization (approximately 16 h) at 42°C, membranes were washed in 2X SSC, 0.1% SDS for 30 min at room temperature.

Blots were then placed in plastic wrap and exposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) for approximately 4 h. Screens were scanned by a phosphorimager and visualized with ImageQuaNT™ software (Molecular Dynamics). If further washing was necessary to remove nonspecific signal, membranes were washed for 15 min twice with 2X SSC, 0.1% SDS at 50° C. Some were washed further with 0.1X SSC, 0.1% SDS at 50°C for 30 min. Membranes were then exposed to the phosphor screen overnight or for several days. Between probing for c-jun and 18S mRNA, membranes were stripped with 50% formamide, 6X SSPE (1X SSPE = 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) for 30 to 45 min at 65°C.

Signal densities were quantitated with ImageQuaNT using volume quantitation of equal areas for any given signal. Background correction was computed by the local average method. Intensity of the c-jun signal relative to the 18S signal was calculated, to assure that comparisons were made between equal quantities of RNA.

Statistical Analysis

Difference in ratio of luteal c-jun/18S RNA for saline or GnRH-treated heifers was determined by student's t-test with Statgraphics (STSC, Rockville, MD). Differences in both plasma oxytocin and serum progesterone were analyzed by repeated measures ANOVA using the general linear model of SAS (1993). Treatment (saline or GnRH), animal(treatment), time and treatment × time were used as the sources of variation. Treatment was tested for significance using animal(treatment) as the error term. Differences among mean plasma concentrations of oxytocin at sampling times after $PGF_{2\alpha}$ injection were

determined by Least Significant Difference test. Differences among mean serum concentrations of progesterone were determined by the contrast procedure of SAS. Differences in peak oxytocin concentrations between treatments, as well as tissue oxytocin concentrations were determined by t-test using Statgraphics. Statistical consultation was provided by Dr. David Thomas, Dept. of Statistics, Oregon State University.

RESULTS

Administration of $PGF_{2\alpha}$ to beef heifers on day 8 of the estrous cycle increased luteal expression of c-jun at 60 min after injection when compared with control animals (Fig. 4). A major transcript was found at 2.7 kb, and a minor transcript was found at approximately 3.6 kb. When the amount of major transcript was quantitated and expressed relative to signal intensity for 18S, treated animals showed a 10- to 20-fold higher induction of c-jun than control animals (Fig. 5). However, GnRH administration on day 2 was not able to alter this $PGF_{2\alpha}$-induced c-jun expression at 60 min after injection, either positively or negatively (Fig. 6) when normalized to signal for 18S rRNA (Fig. 7). Plasma oxytocin increased significantly after $PGF_{2\alpha}$ injection, but there were no differences between saline and GnRH-treated animals (Fig. 8). The mean 30 and 35 min plasma oxytocin samples were not significantly different from the time 0 sample ($p>0.05$), indicating that the oxytocin levels had returned to baseline by 30 min after $PGF_{2\alpha}$ injection. Sample means at all other times were significantly different from the time 0 mean ($p<0.01$). Analysis of peak plasma concentrations of oxytocin (pg/ml) for individual animals in the saline vs. GnRH treatments revealed no difference between injections (saline, 129.1±36.5; GnRH, 124.5±23.5;

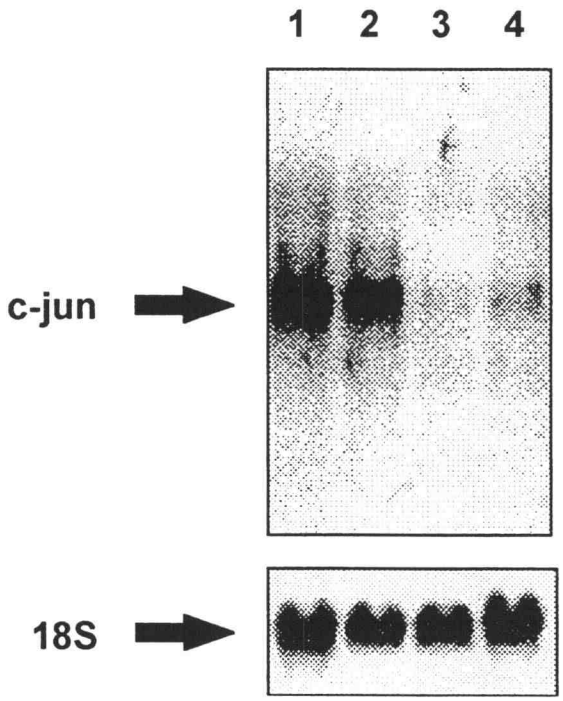

Figure 4. Northern blot of c-jun mRNA from day 8 bovine corpora lutea. Lanes 1 and 2: animals received an injection of 500 μg cloprostenol ($PGF_{2\alpha}$) 60 min prior to removal of corpus luteum. Lane 3: animal received no injection. Lane 4: animal received injection of saline 60 min prior to removal of corpus luteum. 18S rRNA was probed to determine equality of loading.

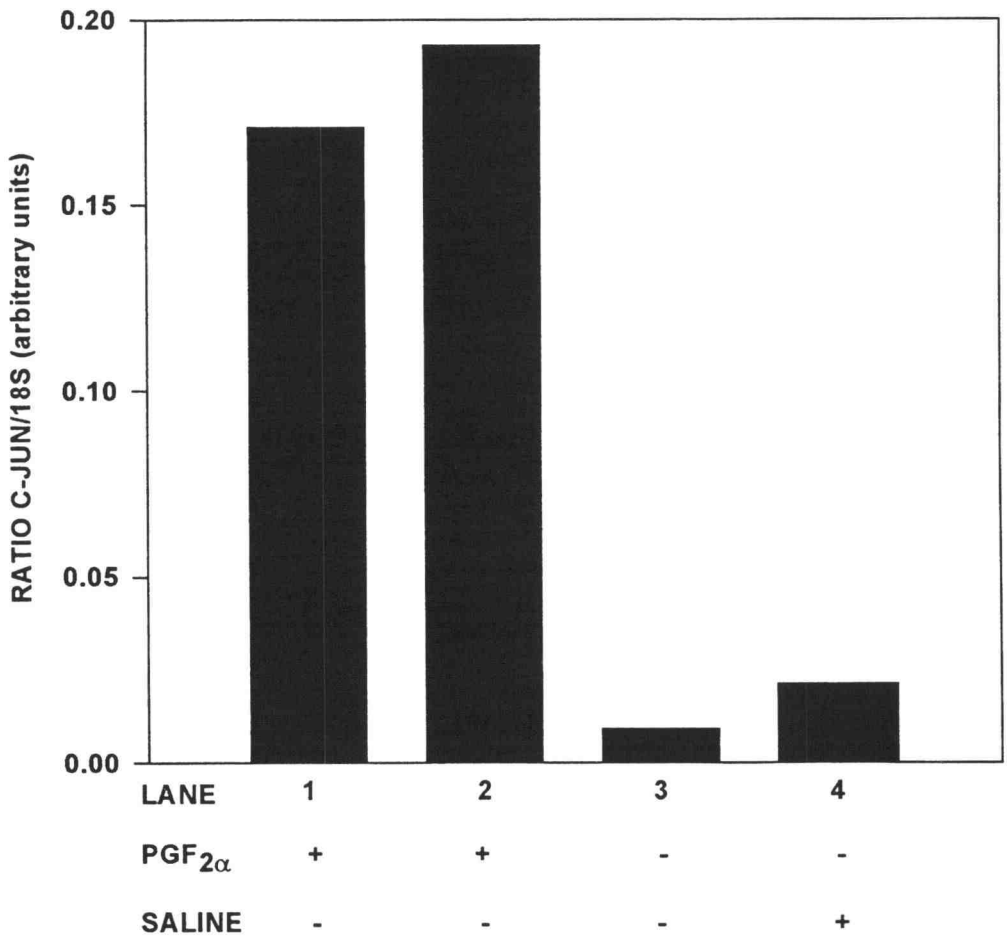

Figure 5. Density of c-jun mRNA signal normalized to the 18S signal for each lane of Fig. 4. Quantitation was performed using volume quantitation of equal areas for a specific signal. Background was corrected for by subtraction of the local average around the area quantitated. The legend below the graph indicates treatment received by individual beef heifers 60 min prior to corpus luteum removal.

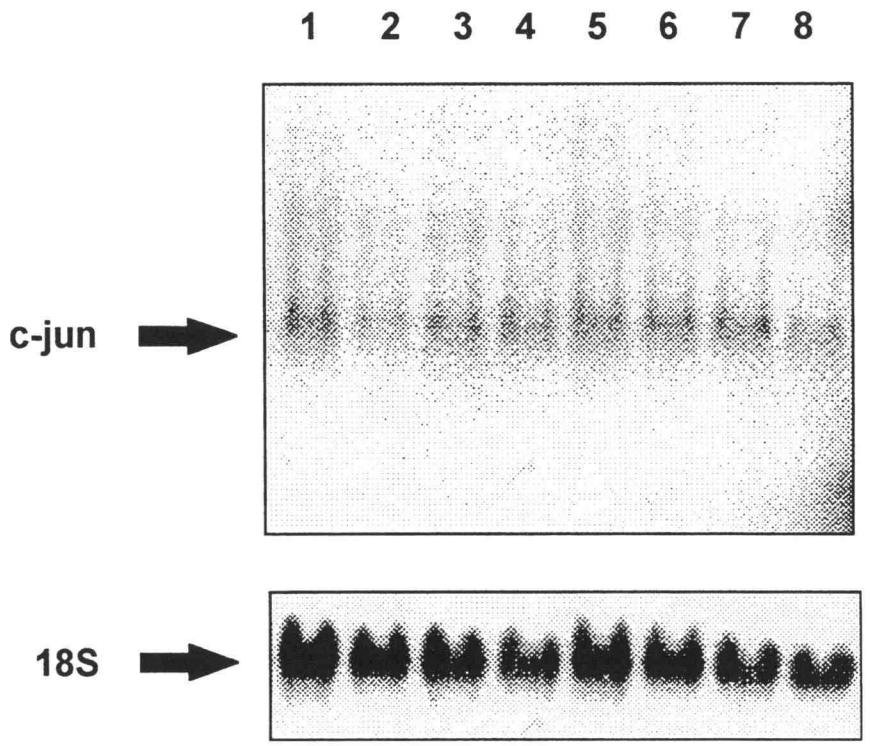

Figure 6. Northern blot of c-jun mRNA from day 8 bovine corpora lutea 60 min after injection of $PGF_{2\alpha}$. Animals had received an injection of saline (lanes 1-4) or GnRH (lanes 5-8) on day 2 of the estrous cycle. 18S rRNA was probed to determine equality of loading.

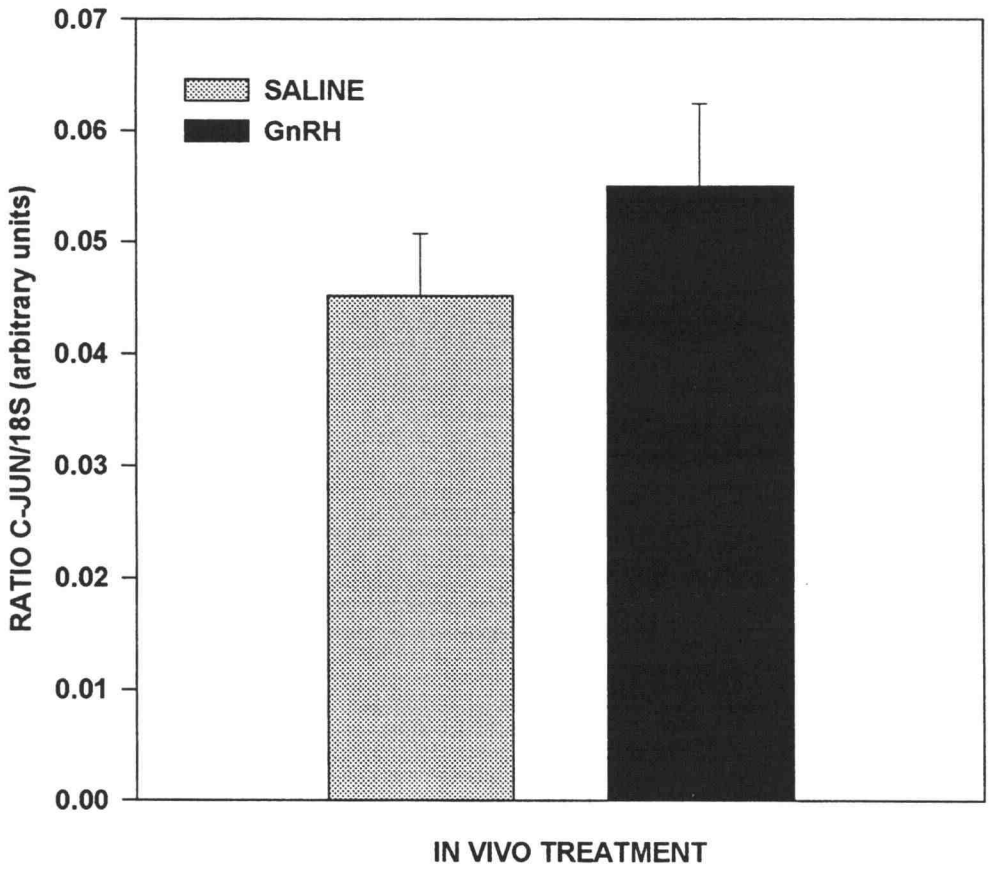

Figure 7. Mean (±SE) densities of c-jun mRNA signal normalized to the 18S signal for Fig . 6. "Saline" represents corpora lutea from animals receiving saline injection on day 2 of the cycle (lanes 1 - 4) and "GnRH" those receiving GnRH injection on day 2 (lanes 5 - 8). All animals received an injection of $PGF_{2\alpha}$ 60 min prior to corpus luteum removal. There was no alteration in the $PGF_{2\alpha}$-induced c-jun expression by early administration of GnRH.

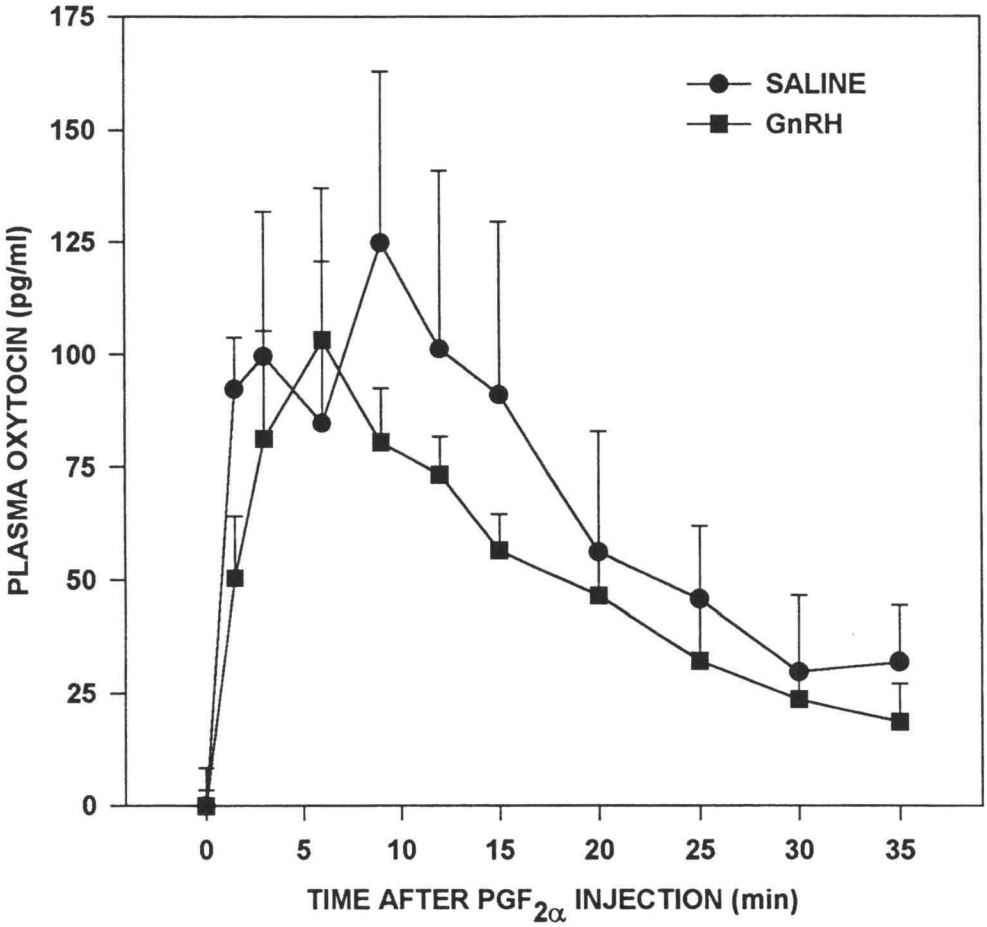

Figure 8. Plasma oxytocin concentrations in beef heifers on day 8 of the estrous cycle following $PGF_{2\alpha}$ injection at time 0. "Saline" and "GnRH" indicate treatments administered on day 2 of the estrous cycle (n = 4 animals/group).

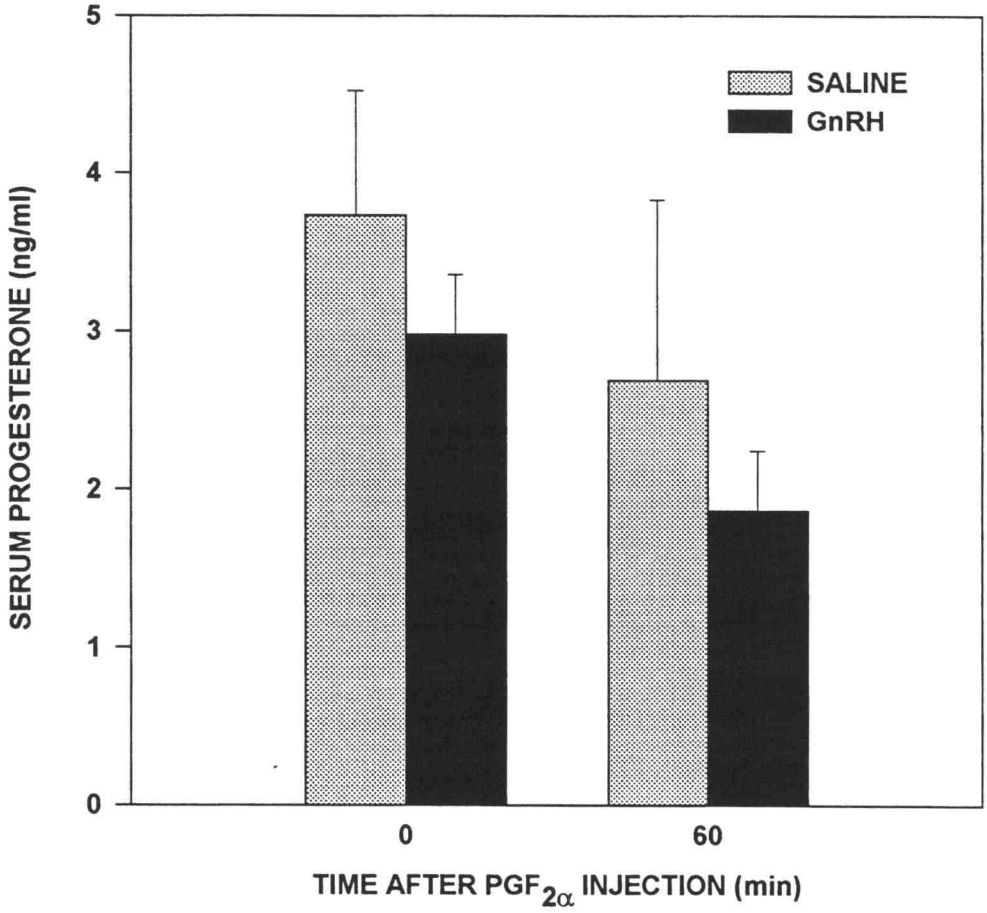

Figure 9. Serum concentrations of progesterone at 0 and 60 min after $PGF_{2\alpha}$ injection into beef heifers on day 8 of the estrous cycle. Gray bars indicate those animals that had received saline injection on day 2, black bars those that received GnRH injection. There was no effect of GnRH injection, however, $PGF_{2\alpha}$ injection significantly decreased progesterone concentrations in both saline and GnRH-treated animals by 60 min after injection ($p<0.05$).

$p>0.05$; data not shown). Peak oxytocin concentrations for all animals occurred between 3 and 12 min after $PGF_{2\alpha}$ injection. Oxytocin in luteal tissue (ng/g tissue) removed 60 min after $PGF_{2\alpha}$ also did not change due to GnRH treatment (saline, 24.07±8.6; GnRH, 19.31±6.0; $p>0.05$; data not shown). Serum concentrations of progesterone (ng/ml) significantly declined by 60 min after $PGF_{2\alpha}$, compared to the zero time sample in both groups (saline 0 min 3.73±0.79, 60 min 2.69±1.1; GnRH 0 min 2.98±0.38, 60 min 1.87±0.38; $p<0.05$; Fig. 9) but GnRH injection did not alter progesterone concentrations ($p>0.05$). However, there was a trend for serum concentrations of progesterone to be lower in GnRH-treated than in control heifers.

DISCUSSION

The potential relevance of the newly discovered mitogen-activated protein kinase cascade as well as its induction and activation of transcription factors such as c-jun have been examined only recently in domestic animals. The induction of c-jun in the CL by $PGF_{2\alpha}$ found in this experiment seems to be the first report of this phenomenon with the exception of the rat (Khan et al., 1993, 1994). The size of the transcripts is consistent with those reported for other steroidogenic tissues. In the rat corpus luteum, a major transcript at 2.6 kb and a minor transcript at 3.5 kb have been found (Khan et al., 1993). In bovine adrenal tissue, the major transcript is found at 2.7 kb and a minor transcript at 3.4 kb (Dr. Mirjana Cesnajaj, National Institutes of Health, Bethesda, MD, personal communication). Additionally, mouse Y1 adrenocortical cells show transcripts for c-jun at 2.7 and 3.6 kb (Kimura et al., 1993). The present study has shown message for c-jun at 2.7 (major transcript) and 3.6 kb (minor transcript) in the bovine corpus luteum (Fig. 4).

The induction of c-jun mRNA 60 min after *in vivo* $PGF_{2\alpha}$ injection also agrees with the published reports of Khan et al. (1993, 1994). Thirty minutes after a second injection of $PGF_{2\alpha}$, c-jun was up-regulated in both day 7 rat corpus luteum and extraluteal tissues (Khan et al., 1993). A time course experiment revealed that c-jun message increased by 15 min after injection of $PGF_{2\alpha}$, and remained high at 30 min and until at least 2 h post-injection. A fourfold induction of c-jun by $PGF_{2\alpha}$ was also reported (Khan et al., 1994). In the present study a 10- to 20-fold induction of c-jun was found in CL removed 60 min after $PGF_{2\alpha}$ injection compared to controls (Fig. 5). The reason for the difference in the amount of c-jun induction in the present study and that of Khan et al. (1994) cannot be fully ascertained. One possible explanation is that Khan et al. (1994) examined time points of 15, 30 and 120 min after injection of $PGF_{2\alpha}$ while the present study utilized CL removed 60 min after injection. It is possible that a "peak" of c-jun induction occurred sometime between 30 and 120 min post-injection and thus time point differences were not observed in the study by Khan et al. (1994), yet the present study may have examined induction of c-jun closer to its "peak". A time course experiment utilizing several additional time points post-injection would be necessary to substantiate this idea. Potential differences between species cannot be ignored, however, nor can small differences in detection techniques that may disallow direct comparison between experiments.

Preliminary reports have indicated that the MAP kinase cascade is indeed active in corpora lutea of domestic animals (Davis et al., 1995; Hildebrandt et al., 1995), and that protein kinase C activates this pathway in these species (Davis et al., 1995). Because this cascade is known to activate the early response genes c-fos and c-jun, and $PGF_{2\alpha}$ is known to act through activation of PKC in the CL, our finding fits into this larger picture of luteal cell function. Khan et al. (1993, 1994) proposed that the induction of c-jun may be an event necessary for

luteolysis, as it certainly is up-regulated during $PGF_{2\alpha}$-induced luteal regression. Thus, in the present study, we utilized this induction of c-jun expression as an indicator to examine whether early administration of GnRH could alter the response of the corpus luteum to a luteolytic stimulus. Early administration of GnRH, however, did not alter $PGF_{2\alpha}$-induced expression of c-jun in the bovine CL in this experiment (Figs. 6 and 7). There are several possible explanations for this occurrence. One is simply that any effect exogenous GnRH may have on the CL did not affect those pathways that involve expression of this early response gene in luteal cells. Additionally, expression of c-jun at the time point after $PGF_{2\alpha}$ injection (60 min post-injection) may not have been altered, but it is possible that the rise in c-jun message or its decline to baseline quantities was. Because the study was not a time course experiment, changes in the rate of c-jun induction, rather than in the amount of induction alone, cannot be ascertained at the present time. It is also possible that c-jun is differentially up- or down-regulated in large or small luteal cells, a process that could be affected by GnRH, but not found in whole CL extract.

Early administration of GnRH is thought to increase the large luteal cell to small luteal cell ratio in the bovine CL (Mee et al., 1993). Because oxytocin, a hormone important in luteal regression, is derived from the large cells of the CL, it is possible that administration of GnRH could alter oxytocin secretion from the CL. In addition, the release of oxytocin from the bovine CL after injection of $PGF_{2\alpha}$ can be used as another indicator of luteolytic potential of GnRH-exposed corpora lutea. In the present study, however, neither concentrations of plasma oxytocin (Fig. 8) nor oxytocin in luteal tissue 60 min after injection of $PGF_{2\alpha}$ were significantly altered by early administration of GnRH. In contrast, Whitmore (1995) showed a reduction in naturally-secreted luteal oxytocin on days 12 and 14 in intact ewes that had been injected with GnRH on days 2 and 3

of the cycle. However, other studies that have examined the luteolytic potential of the CL after exogenous GnRH, LH or hCG have only examined whether these compounds can affect the decline in progesterone production by the CL. In the present study, serum concentrations of progesterone did fall by 60 min after injection of $PGF_{2\alpha}$, but at neither 0 nor 60 min after $PGF_{2\alpha}$ were the serum concentrations of progesterone significantly affected by early administration of GnRH (Fig. 9). Bolt (1979) found that attempts to alter the luteolytic effect of $PGF_{2\alpha}$ treatment on day 10 with hCG administered early in the estrous cycle to ewes were variable and for the most part unsuccessful. But when hCG was given on days 9 to 10, $PGF_{2\alpha}$ injection on day 10 was unable to induce luteal regression in ewes. This finding, however, contrasts with those of several others. In cows, Litch and Condon (1988) followed a similar protocol but found that hCG did not alter $PGF_{2\alpha}$-induced regression of the CL, and that *in vitro* progesterone production was decreased in hCG/$PGF_{2\alpha}$-treated animals compared to non-injected controls. In mid-cycle cows (González-Menció et al., 1977) and ewes (Sasser et al., 1977) infusion of LH for 10 or 12 h did not affect $PGF_{2\alpha}$-induced luteal regression when $PGF_{2\alpha}$ was injected during the infusion. In both studies, decline in serum progesterone was similar in control and treated animals. Interestingly, Macmillan et al. (1985) found that injection of the GnRH agonist Buserelin at various times during diestrus, 15 min, 24 h or 72 h before injection of $PGF_{2\alpha}$ did not alter functional luteolysis, but prevented or slowed structural luteolysis. Plasma concentrations of progesterone declined similarly in GnRH-pretreated and control animals after injection of $PGF_{2\alpha}$. In animals injected with $PGF_{2\alpha}$ alone, however, palpated CL were detectably smaller by 24 h post-injection, while those also treated with GnRH did not change in size until at least 3 days after $PGF_{2\alpha}$ injection. In the present study, serum concentrations of progesterone also declined in both control and GnRH-treated animals after

$PGF_{2\alpha}$ injection. However, alteration of structural changes could not be assessed because the CL were removed 60 min after injection of $PGF_{2\alpha}$.

To summarize, both c-jun gene expression and plasma concentrations of oxytocin increased following injection of $PGF_{2\alpha}$ into beef heifers on day 8 of the estrous cycle, while serum concentrations of progesterone declined. These are expected responses of the corpus luteum when exposed to a luteolytic dose of $PGF_{2\alpha}$. However, injection of GnRH on day 2 of the cycle was unable to alter these $PGF_{2\alpha}$-induced events. Alteration of luteal function after early administration of GnRH does not appear to involve $PGF_{2\alpha}$-responsive cellular mechanisms.

GENERAL CONCLUSIONS

The present study has examined the effect of exogenous GnRH on the corpus luteum at various stages of the luteal life span. Injection of GnRH on day 2 of the estrous cycle allows for modification of the hormonal milieu of the corpus luteum during its development. Examination of the responses of luteal slices or cells to hormonal stimuli on day 7 demonstrated how early injection of this decapeptide can alter luteal performance as it nears the time of full functionality. Oxytocin secretion in response to $PGF_{2\alpha}$ is greatest on day 8, and thus the beginning of the luteolytic process could also be explored.

The role of GnRH in alteration of progesterone production by the CL still seems to be somewhat enigmatic. In the first experiment, GnRH was able to inhibit both LH and cAMP-stimulated progesterone production by bovine luteal slices. However, when a cell culture system was used in Exp. 2, no effect of GnRH was found. Mee et al. (1993) explained inhibition of hormone-stimulated progesterone production from luteal slices by showing an increased large luteal cell to small luteal cell ratio in GnRH-exposed corpora lutea. Thus, the presence of more large cells and fewer small cells does not allow for the same amount of stimulation as in control CL, because the small cells are the ones considered to be responsive to LH. However, in neither the present study nor that of Mee et al. (1993) is the lack of change in basal progesterone production by the luteal slices easily explained. Because large cells produce more progesterone in the absence of hormonal stimuli, it should be expected that the basal progesterone production would actually increase at the same time the hormone-stimulated progesterone decreased. This was not the case, however. Mee et al. (1993) did find that serum progesterone was increased after early administration of GnRH,

and stated that this finding supported their cell-size hypothesis, because the main source of progesterone *in vivo* can be considered to be basal progesterone from the large cells. However, in the present studies no change in serum concentrations of progesterone was found on days 7 or 8 after injection of GnRH on day 2 of the cycle. Various studies have found no change, increases or decreases in serum progesterone after injection of GnRH. Thus the effect of GnRH on the corpus luteum is somewhat variable, and while the data presented herein are consistent with the hypothesis of Mee et al. (1993), other factors influencing progesterone production by the CL after GnRH injection cannot be fully excluded. These can include changes in intracellular functioning at a point distal to the accumulation of cAMP in the luteal cell.

In considering other hormones, it does not appear that GnRH alters $PGF_{2\alpha}$-induced oxytocin secretion nor the decline in serum progesterone indicative of ebbing luteal function. Thus one practical application of this research might be that in the event of a misdiagnosis of follicular cysts. Use of GnRH is not detrimental even in the absence of follicular cysts, because if the animal does not return to estrus as expected $PGF_{2\alpha}$ can subsequently be administered. The GnRH-exposed corpus luteum will behave normally in response to this luteolytic stimuli. The use of GnRH and $PGF_{2\alpha}$ has already been suggested as an estrous synchronization tool that allows for more precise timing of synchronization (Thatcher et al., 1989b; Twagiramungu et al., 1992). Thus it actually seems an advantage that GnRH does not alter $PGF_{2\alpha}$-induced luteal regression.

One avenue of research that is currently unexplored is the study of gene regulation in the corpus luteum after GnRH injection early in the estrous cycle. It would be of interest to know what genes are up- or down-regulated by early GnRH administration so that the cellular mechanisms by which GnRH acts,

rather than mechanisms in place after it has already had its effect, can be ascertained. A technique such as differential display PCR could possibly be utilized, comparing genes unique to early corpora lutea of controls relative to those of CL exposed to GnRH. Recently this technique has been used successfully to examine genes unique to ovulation (Espey et al., 1995) and the luteinization process (Leers-Sucheta and Melner, 1995). In the present report, regulation of the c-jun gene has been examined subsequent to $PGF_{2\alpha}$, or GnRH and $PGF_{2\alpha}$ injections. Luteal regression is a complex process that is certainly not fully understood, especially at the molecular level. Thus, further exploration of gene regulation during luteolysis is warranted, including both early response genes and delayed response genes. The present study supports the findings of Khan et al. (1993, 1994) in showing that expression of the early response gene c-jun is up-regulated during $PGF_{2\alpha}$-induced luteolysis in the rat and now, the cow. Additionally, study of protein(s) produced by gene up-regulation after $PGF_{2\alpha}$ could lead to further comprehension of the luteolytic process. Perhaps examination of gene regulation or the activation/phosphorylation of the c-jun protein itself during the course of normal luteal regression (near the end of the estrous cycle) could provide insight into its physiological role.

The corpus luteum is a unique, transient endocrine organ, and it possesses qualities that have made it the focus of many research studies. It grows rapidly from the ovulated follicle, produces quantities of hormone that can sustain at least the beginnings of pregnancy and it regresses when it is no longer needed, such as at end of a non-fertile estrous cycle. Study of this tissue has provided numerous insights into the control of reproductive function. Many more investigations, especially at the level of gene regulation in the corpus luteum, remain to be completed.

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