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Title: OXYTOCIN SYNTHESIS AND RELEASE BY THE BOVINE CORPUS LUTEUM

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Four experiments were conducted to determine oxytocin concentration of bovine luteal tissue at different stages of the estrous cycle and to study the effects of prostaglandins $F_{2\alpha}$ ($PGF_{2\alpha}$), E_2 (PGE_2), luteinizing hormone (LH), cycloheximide, colchicine and cytochalasin B on oxytocin synthesis and release in vitro. In experiment 1, luteal oxytocin concentrations (ng/g) in beef heifers increased from 414 ± 84 on day 4 to 2019 ± 330 on day 8 and then declined to 589 ± 101 on day 12 and 81 ± 5 on day 16 of the cycle. Prostaglandin $F_{2\alpha}$ induced a significant in vitro release of luteal oxytocin on day 8 but not on days 12 or 16, while PGE_2 and LH had no

effect on oxytocin release at any stage of the cycle studied. Total oxytocin concentration (incubation medium + tissue) increased twofold after 2 h of incubation. In experiment 2, six beef heifers were used to investigate *in vitro* effects of 10, 20 and 40 ng $PGF_{2\alpha}$/ml of medium on oxytocin release from day 8 luteal tissue. A significant linear dose-response relationship was observed indicating oxytocin release was dose-dependent. In experiment 3, the effects of cycloheximide on oxytocin synthesis as well as $PGF_{2\alpha}$-induced oxytocin release from day 8 bovine luteal tissue was investigated. Although, cycloheximide inhibited incorporation of labeled leucine into protein by more than 90%, it did not affect processing of prohormone or prostaglandin $F_{2\alpha}$-induced release of this nonapeptide from luteal tissue. No incorporation of labeled leucine was detected in oxytocin. In experiment 4, the effects of cytochalasin B and colchicine on oxytocin synthesis and release from day 8 bovine luteal tissue were investigated. Neither colchicine nor cytochalasin B inhibited oxytocin synthesis or release while $PGF_{2\alpha}$ caused a significant release of oxytocin that was not inhibited by colchicine. These studies indicate that maximal oxytocin concentrations in bovine luteal tissue occur during the early luteal phase of the cycle. Luteal oxytocin secretion *in vitro* can be induced by $PGF_{2\alpha}$ whereas PGE_2 and LH have no effect on oxytocin secretion. Increased oxytocin concentration due to short term incubation represents a post-translational processing of an oxytocin prohormone because it occurred in the absence of incorporation of labeled leucine and during inhibition of *de novo* protein synthesis.

Oxytocin Synthesis and Release by the Bovine Corpus Luteum

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To my wife Afaf, for her immense patience,
magnificent devotion, encouragement and love,
and to my son Mohamed and my daughter Reim,
for making life so wonderful and enjoyable,
I dedicate this thesis

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LITERATURE REVIEW

Hormonal Control of Corpus Luteum Function During the Estrous Cycle and Pregnancy

Understanding the basis for estrous cycles and the maintenance of pregnancy in domestic animals requires an appreciation of the factors that regulate corpus luteum function.

The function of the corpus luteum in ruminants during the estrous cycle and pregnancy is regulated by a complex interaction of hormones from several sources, including the pituitary gland, uterus and placenta. These hormones may act directly, or through indirect mechanisms on luteal function and are either stimulatory (luteotropic) or inhibitory (luteolytic) to progesterone synthesis and secretion, which are universally utilized as measures of the state of luteal function.

In addition, factors synthesized and secreted within the ovary may influence corpus luteum function. The existence of a paracrine mechanism for regulation of luteal function is becoming increasingly evident. The corpus luteum of domestic animals and primates is composed of at least two cell types that differ in their function and response to hormonal stimuli and which may interact to regulate luteal function.

In this section, hormonal regulation of the bovine and ovine estrous cycles as well as corpus luteum function during early pregnancy will be discussed. Special consideration will be devoted to the review of follicular growth as well as effects and mechanisms of action of luteinizing hormone (LH), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), embryonic luteotropins and paracrine factors on luteal function.

General Features of the Estrous Cycles of the Ewe and Cow

The estrous cycle is shorter in ewes (17 days) than in cows (21-22 days). Duration of standing estrus is 24-36 h in the ewe and 18-19 h in the cow. Ewes normally ovulate near the end of estrus but time of ovulation varies from as long as 11 h before to 7 h after the end of estrus. Cattle ovulate on the average at 28-32 h after the begining of estrus, which normally corresponds to 12 h after the end of standing estrus.

Estrous cycles in the cow and ewe are characterized by a long luteal phase. This is the period during which an active corpus luteum resides in the ovary. In contrast, the follicular phase, the period from corpus luteum regression to the following ovulation is apparently short (2 days in ewes and 4 to 5 days in cows). However, the presence of antral follicles throughout the luteal phase suggests that the real duration of the follicular phase is longer than 2 to 5 days, if one considers that the follicular phase refers to the period from antral follicle formation to ovulation. Therefore, the luteal phase in these species may partially overlap the true follicular

phase, obscuring the relationship between the hormones that regulate luteal function and follicular growth (Hafez et al., 1980).

Hormonal Control of Folliculogenesis

Because follicular growth and recruitment of ovulatory follicles are an integral part of the estrous cycle, it is deemed appropriate to mention the factors that regulate folliculogenesis. Most of these factors are also involved in the regulation of the estrous cycle.

Primordial follicles, with their single oocyte surrounded by a squamous follicular epithelium, are established in the ovary during fetal development. After birth oocytes are arrested in the dictyate stage of the first mitotic prophase. This resting stage is called the dictyate nucleus. Primary follicles enter a pool of growing follicles during adulthood in response to an undefined stimulus. However, a vast majority of these growing follicles degenerate by a process known as atresia. Of the approximately 150,000 primordial follicles present at birth in heifers (Erickson, 1966), less than 100 will mature and ovulate during the life time of an average animal (Hansel and Convey, 1983).

Primordial follicles enter a growing phase when the follicular cells proliferate and form several layers of granulosa cells. As the follicle grows it is displaced toward the center of the ovary, the theca layer differentiates into two layers; interna and externa, and the oocyte aquires a distinct zona pellucida. Growing follicles form fluid filled antra by the coalesence of small fluid filled cavities

between follicular cells (Hansel and Convey, 1983).

Factors controlling growth, atresia and selection of the ovulatory follicle(s) are not completely understood. Luteinizing hormone and follicle stimulating hormone (FSH) are released concomitantly at or near the onset of estrus in cows (Akbar et al., 1974) and ewes (Pant et al., 1977) and approximately 24 h after the preovulatory gonadotropin surge, but before ovulation, there is a second increase in serum concentrations of FSH in ewes (Pant et al., 1977) and cows (Dobson, 1978; Ireland and Roche, 1982). This increase may play a role in recruitment of preantral follicles. Cahill et al. (1981) found a high correlation between the magnitude of this peak in serum FSH concentration and number of antral follicles present in the ovary 17 days later. Follicle stimulating hormone also stimulated granulosa cell mitosis and follicular fluid formation. In addition, 17β-estradiol produced by the granulosa cells enhanced this FSH-mitotic effect. Follicle stimulating hormone also induces granulosa cell sensitivity to LH by increasing the number of LH receptors (Hafez et al., 1980).

Intraovarian factors may also control the growth of primordial follicles and the selection of those destined to ovulate. Meiosis of the dictyate nucleus never resumes normally before a gonadotropin ovulatory surge. However, in all mammalian species studied, when the oocyte was removed from the antral follicle and cultured in a gonadotropin-free medium, it spontaneously resumed meiosis up to metaphase I or metaphase II, the stage normally attained at the time

of ovulation (Thibault, 1977). Culture of the oocyte with granulosa or theca cells or in a medium that contained follicular fluid extracts, showed that the maintenance of the oocyte nucleus in a dictyate state resulted from the inhibitory effect of the granulosa cells on the oocyte (Foote and Thibault, 1969; Tsafriri and Channing, 1975). The role of the LH-FSH ovulatory surge is to cause loosening of granulosa cell junctions and to suppress production of a meiotic-inhibiting factor by granulosa cells (Hafez et al., 1980).

Removal of the corpus luteum of cows (Hammond and Bhattacharya, 1944) and ewes (Smeton and Robertson, 1971) resulted in ovulation in 48 to 72 h. However, large follicles present in ovaries at the time of corpus luteum removal (Smeton and Robertson, 1971) or regression (Ireland and Roche, 1982) become atretic, while newly recruited follicles develop and subsequently ovulate. Removal of the corpus luteum or its regression may therefore, eliminate a local inhibitor, or it may increase gonadotropin stimulation leading to final growth and maturation of the preovulatory follicles, whose development coincided with luteal regression and a preovulatory gonadotropin surge.

Intraovarian factors may interact within themselves and with hypothalamic and other factors to regulate folliculogenesis and ovulation. Synthesis of hypothalamic hormones and their receptors in ovarian tissue as well as the presence of unique peptides in the ovary that bind gonadotropin receptors, and the existence of uterine factors that inhibit FSH secretion give credence to this concept

(Hansel and Convey, 1983).

Hormonal Control of the Estrous Cycle in the Ewe and Cow

The estrous cycle is controlled by the interaction of FSH, LH, estrogen and progesterone. These hormones are common to most domestic animals; however, their secretory patterns and relative effects vary among different species. These differences lead to variations in the length of follicular and luteal phases of the cycle as well as differences in duration of estrus.

Patterns of hormonal changes during the estrous cycle in the ewe and cow are similar. The follicular phase of the cycle is characterized by rapidly decreasing levels of progesterone due to luteal regression, a peak of estradiol and a slight but significant increase in LH levels, which may be important in inducing follicular maturation and the proestrous rise in estradiol (Hansel and Convey, 1983). Estradiol increased in ovarian venous (Bjersing et al., 1972; Baird and Scaramuzzi, 1976) and in systemic blood (McCracken et al., 1971; Hansel et al., 1973) during the preovulatory period reaching a peak during estrus. Removal of the negative feedback influence of progesterone and the increase in estradiol concentration exert a positive feedback effect on the hypothalamus-hypophyseal axis resulting in the ovulatory surge of LH and FSH that occurs approximately 12 h after the onset of estrus in the ewe (Kaltenbach and Dunn, 1980; Hansel and Convey, 1983). The increase in estradiol that occurs during the preovulatory period is clearly the stimulus

that triggers the gonadotropin surge. Exogenous estradiol induced a preovulatory-like surge of LH in ewes (Howland et al., 1971) and cows (Beck and Convey, 1977). In addition, chemical or immunological inhibition of estradiol at proestrus abolished the LH surge in ewes (Fairclough et al., 1976) and cows (Martin et al., 1978). However, a decrease in progesterone is requisite for an estradiol effect on the gonadotropin surge. Estradiol did not exert a positive feedback effect in females bearing a maximally functional corpus luteum (Bolt et al., 1971; Short et al., 1973), and exogenous progesterone blocked the estradiol-induced gonadotropin surge in ewes (Scaramuzzi et al., 1971) and heifers (Kesner et al., 1981). Nevertheless, the mechanism by which estradiol induces a gonadotropin surge is not completely understood. Exogenous estradiol increased the capacity of the pituitary gland to release LH and FSH in response to gonadotropin releasing hormone (GnRH) in ewes and cows (Reeves et al., 1971; Kesner et al., 1981). This capacity was greatest during estrus and least during the luteal phase of the cycle (Convey, 1973). Estradiol also has the ability to prime the response of the anterior pituitary gland to subsequent exposures to GnRH, thereby increasing the quantity of LH and FSH released by a standard dose of GnRH (Crighton and Foster, 1977; Hansel and Convey, 1983). This priming effect was responsible, at least in part, for the marked increase in pituitary sensitivity that occurred during the proestrus period. Estradiol markedly increased the ability of GnRH to prime bovine pituitary cells <u>in vitro</u>, an effect that is inhibited by progesterone

(Padmanabhan et al., 1982). Moreover, consecutive exposures to pulsatile releases of GnRH, under estrogen dominance, progressively increased the magnitude of LH pulses thereby creating the preovulatory surge of LH. Both increased GnRH secretion and increased pituitary responsiveness are necessary for the preovulatory LH and FSH surges (Kesner and Convey, 1982). Termination of preovulatory LH and FSH surges results from refractoriness of the pituitary gland to GnRH (Chakraborty et al., 1974; Kesner and Convey, 1982) and not to depletion of gonadotropin content (Convey et al., 1981).

Increased LH concentrations during the presurge period resulted from increased frequency and decreased amplitude in pulsatile secretion of this gonadotropin in ewes (Baird, 1978) and cows (Rahe et al., 1980). These pulses of LH may stimulate estradiol secretion from preovulatory follicles. Each pulse of LH was followed by an increased concentration of estradiol in ovarian venous blood of ewes (Baird et al., 1976) and exogenous LH increased estradiol secretion from autotransplanted ovaries (McCracken et al., 1971). Preovulatory follicles have been assessed as estrogen active (nonatretic) and estrogen inactive (atretic) based upon histological assessment of granulosa cells and steroid hormone content of follicular fluid (Moor et al., 1978; Carson et al., 1981). Following luteal regression, both estrogen active and inactive follicles are present on the ovaries, but by the time of the LH surge, only estrogen active follicles remain. Ovulatory follicle(s) grow in size and the number

of LH receptors in theca and granulosa cells increases. Consequently these follicles become more responsive to LH and aquire an increased ability to secrete estradiol. During the postovulatory period in heifers, a single estrogen active follicle develops and all other estrogen active follicles regress (Ireland and Roche, 1983). This follicle is probably the source of increased estradiol secretion at this time of the estrous cycle (Glencross et al., 1973; Hansel et al., 1973).

Mechanism of Ovulation

Preovulatory follicles undergo three major changes during the ovulatory process. These include: disruption of cumulus cell cohesiveness among the granulosa layer, cytoplasmic and nuclear maturation of the oocyte and thinning and rupture of the external follicular wall.

The freeing of the oocyte inside the follicle is the only known response directly attributable to gonadotropic action. In vitro dissociation of cumulus cells is exclusively obtained by FSH and LH (Thibault et al., 1975). The role of the LH-FSH ovulatory surge is to cause loosening of granulosa cell junctions, suppress production of oocyte meiotic-inhibiting factor by granulosa cells and allow the oocyte to resume the meiotic division as discussed previously.

The preovulatory gonadotropin surge also induces ovulation by a cascade of biochemical changes. It has been shown to cause an immediate and temporary rise in steroid levels due to an increased

secretion of progesterone. Later $PGF_{2\alpha}$ and prostaglandin E_2 (PGE_2) secretion was also augmented. Inhibition of either progesterone (Lipner and Greep, 1971) or prostaglandin (Armstrong, 1975) synthesis prevented ovulation. Enhancement of steroid secretion and the change in the ratio of estradiol to progesterone that follow the gonadotropin surge were easily detectable in follicular fluid (Gerard et al., 1979). These changes were barely detectable in ovarian venous blood and undetectable in systemic blood (Eiler and Nalbandov, 1977). It has been postulated that the role of progesterone is to stimulate the activity of collagenase and other proteolytic enzymes (Rondell, 1970), which degrade connective tissue in the follicular wall.

Prostaglandins play a basic role in follicular rupture, and their action is exerted at the level of the albuginea and follicular epithelium. They induce ovulation by increasing the activity of proteolytic enzymes that cause rupture of the follicle and release of the oocyte (Espey, 1978). Inhibition of prostaglandin synthesis prevented ovulation but not luteinization (Yang et al., 1973; Lau et al., 1974; Armstrong and Zamecnik, 1975). As a result the oocyte remained inside the luteinized follicle (Osman and Dullaart, 1976).

Prostaglandin $F_{2\alpha}$ stimulated synthesis, release and activation of a collagenase-like ovulatory enzyme (Marsh and LeMaire, 1973; LeMaire and Marsh, 1975). It also contributed to the rupture of the lysosomes of epithelial cells at the follicular apex and stimulated the production of plasminogen activator, thus increasing plasmin

activity that converted procollagenase to collagenase (Espey, 1980) and is generally involved in cell migration and mixing of theca and granulosa cells during corpus luteum formation.

Prostaglandin E_2 has also been shown to stimulate production of plasminogen activator (Strickland and Beers, 1976) and the remodeling of follicular layers, terminating in corpus luteum formation (Hafez et al., 1980).

Formation of the Corpus Luteum

Following ovulation the wall of the follicle gradually thickens due to hypertrophy and hyperplasia of the theca and granulosa cells. Rapidly proliferating cells fill the remaining cavity and begin to secrete progesterone. The resulting corpus luteum continues to increase in size and weight and attains full growth and function 7-9 days after ovulation in the ewe (Duncan et al., 1960) and 12 days postovulation in the cow (Erb et al., 1971).

The size of the corpus luteum is highly correlated with its ability to secrete progesterone. Progesterone and 20β-hydroxyprogesterone content of bovine corpus luteum (Hafs and Armstrong, 1968) and progesterone in systemic blood (Hansel et al., 1973) increase to maximal levels at approximately day 10. Progesterone prepares the endometrium for implantation and maintains pregnancy.

Luteotropic Effect of LH

The first indication that LH possessed luteotropic properties was provided by Mason et al. (1962) who demonstrated its stimulatory effect on *in vitro* progesterone synthesis by bovine luteal slices. Since then much evidence has accumulated indicating that LH is the luteotropic hormone in both the cow and ewe (see Hansel et al., 1973; Rothchild, 1981; Niswender et al., 1985). Exogenous LH injections prolonged the life span of bovine (Donaldson and Hansel., 1965b) and ovine (Karsch et al., 1971) corpora lutea during the estrous cycle, increased progesterone concentration in the plasma of hysterectomized ewes and cows (Brunner et al., 1969; Carlson et al., 1971) and prevented the luteolytic effects of oxytocin administered early during the bovine estrous cycle (Donaldson et al., 1965). In addition, simultaneous injections of a potent antibovine LH serum produced in mares caused a significant reduction in corpus luteum weight and progesterone content in intact heifers (Snook et al., 1969) and inhibited stimulatory effects of LH on progesterone synthesis (Hansel, 1971).

Furthermore, the secretion of progesterone depends on continuous luteotropic support from the pituitary gland. Progesterone synthesis and secretion ceased and the corpus luteum regressed within a few days following hypophysectomy in the ewe. However, maintenance of the corpus luteum in hypophysectomized pregnant and nonpregnant ewes occurred if crude pituitary extracts containing LH and FSH activity were constantly infused (Kaltenbach et al., 1968a,b). In addition

LH, but not prolactin or FSH markedly increased progesterone secretion in autotransplanted ovaries in the ewe (McCracken et al., 1971).

Mechanism of Action of LH

At the molecular level LH exerts its action by initiating a cascade of biochemical reactions that lead to increased progesterone synthesis and the expression of a functional corpus luteum (for a recent review see Stormshak et al., 1987).

Luteinizing hormone binds to its receptor in the plasma membrane of the luteal cell. This binding activates adenylate cyclase which convertes adenosine triphosphate (ATP) to cyclic adenosine 3'-5' monophosphate (cAMP) which in turn activates a cAMP-dependent protein kinase. Protein kinase in turn causes phosphorylation of steroidogenic enzymes and other proteins necessary for synthesis and secretion of progesterone. The LH-receptor complex is then internalized, degraded and the LH receptor is recycled via secretory granules and incorporated in the plasma membrane by exocytosis (Niswender et al., 1981).

Mechanism of Luteolysis

In the nonpregnant ewe (Thorburn et al., 1972) and cow (Robinson, 1977), the corpus luteum regresses abruptly 13 to 15 days and 18 days following ovulation, respectively. There is substantial evidence that $PGF_{2\alpha}$ of uterine origin is the agent responsible for

normal regression of the corpus luteum in the ewe and cow (for review see Goding, 1974; Horton and Poyser, 1976; Hansel and Convey, 1983; Stormshak et al., 1987). Corpus luteum regression results in a decline of the secretory activity of the gland (functional luteolysis) followed by degenerative changes (structural luteolysis) such as a decrease in cytoplasmic granulation, a rounding of the cell outline and peripheral vacuolation of the large luteal cells (Hansel et al., 1973; Stormshak et al., 1987).

Functional luteolysis results from short term exposure of the corpus luteum to $PGF_{2\alpha}$ and culminates in reduced progesterone synthesis and secretion. Prostaglandin $F_{2\alpha}$ may induce functional luteolysis by interfering with LH activation of adenylate cyclase activity within luteal plasma membranes (Henderson and McNatty, 1975). Exposure of rat (Lahav et al., 1976), ovine (Fletcher and Niswender, 1982), bovine (Marsh, 1971), human (Hamberger et al., 1979) and nonhuman primate (Stouffer et al., 1979) corpora lutea to $PGF_{2\alpha}$ in vitro resulted in inhibition of gonadotropin induced-adenylate cyclase activity and cAMP production. Furthermore, natural luteolysis in cattle (Garverick et al., 1985), pigs (Ritzhaupt et al., 1986) and primates (Eyster et al., 1985) as well as $PGF_{2\alpha}$-induced luteolysis in ewes (Agudo et al., 1984) and pseudopregnant rats (Khan and Rosberg, 1979) was accompanied by a decrease in basal and(or) LH-stimulated adenylate cyclase activity.

Prostaglandin $F_{2\alpha}$ has also been recently shown to stimulate phosphoinositide metabolism in bovine (West et al., 1986) and rat

(Leung et al., 1986) luteal cells in vitro. This action of $PGF_{2\alpha}$ resulted in generation of inositol 1,4,5-triphosphate and diacylglycerol whose actions as second messengers in target tissues elicit calcium mobilization from the endoplasmic reticulum and activation of protein kinase C, respectively (Berridge and Irvine, 1984; Nishizuka et al., 1984).

Moreover calcium effects on intact luteal cells resembled those of $PGF_{2\alpha}$. Calcium ionophore A23187 inhibited LH-induced cAMP accumulation in rat luteal cells (Dorflinger et al., 1984) and caused an increase in intracellular calcium levels that resulted in inhibition of LH-stimulated progesterone synthesis by small bovine luteal cells (Hansel and Dowd, 1986).

Elevated intracellular calcium levels may result in activation of many enzymes that affect luteal cell function. An increase in the activity of phosphodiesterase, a calcium-dependent enzyme that inactivates cAMP, was noted within 2 h of $PGF_{2\alpha}$ administration to ewes (Agudo et al., 1984). In addition, increased intracellular calcium levels may decrease plasma membrane fluidity by activating phospholipase A_2 which catalyzes release of arachidonic acid from phospholipid (Riley and Carlson, 1985,1986). Degenerative changes in plasma membranes correlated with increased phospholipase A_2 activity were suggested to result from elevations in luteal prostaglandin synthesis. Such changes could accelerate luteal regression in a positive feedback manner and(or) the generation of superoxide anions through the action of lipoxygenases on arachidonic acid (Riley and

Carlson, 1985). Rothchild (1981) proposed that $PGF_{2\alpha}$ whether of uterine or ovarian origin, could stimulate its own production in luteal tissue of all species, thus contributing to the completion of luteolysis in a paracrine fashion.

Chronic exposure to $PGF_{2\alpha}$ for more than 24 h results in structural luteolysis. In addition to the morphological changes listed above, there was a decrease in LH receptor concentration, membrane fluidity and steroidogenic enzyme activity as well as an increase in the activity of lysosomal enzymes (Stormshak et al., 1987).

Prostaglandin $F_{2\alpha}$ may affect luteal tissue directly or indirectly. A direct action of $PGF_{2\alpha}$ on luteal cells is supported by the existence of specific receptors located within the plasma membranes of ovine (Powell et al., 1974a), bovine (Powell et al., 1976; Lin and Rao, 1977), equine (Kimball and Wyngarden, 1974), human (Powell et al., 1974b) and rat (Luborsky-Moore et al., 1979) corpora lutea. Prostaglandin $F_{2\alpha}$ may also affect the corpus luteum indirectly by restricting the blood flow through the luteal vascular bed but such an effect of $PGF_{2\alpha}$ is equivocal (Nett et al., 1976; Niswender et al., 1976).

Endocrine Regulation of $PGF_{2\alpha}$ Secretion

Prostaglandins are secreted by the uterus in sporadic pulses (Thorburn et al., 1973). Mean concentrations of $PGF_{2\alpha}$ in the utero-ovarian venous plasma are elevated between days 12 and 14 of the

estrous cycle in ewes (Silvia et al., 1984) leading to luteal regression and the beginning of a new cycle.

Endocrine regulation of $PGF_{2\alpha}$ secretion in ewes appears to be controlled primarily by estradiol and oxytocin (McCracken et al. 1984). According to this hypothesis the uterotropic actions of progesterone appear to decline as the luteal phase progresses, permitting endogenous estradiol to stimulate oxytocin receptor synthesis in the endometrium. Endogenous luteal oxytocin interacts with its receptor to cause secretion of $PGF_{2\alpha}$ from the endometrium. Luteal regression is initiated as a result of the countercurrent transfer of $PGF_{2\alpha}$ from the uterine vein to the ovarian artery. Further release of oxytocin from the corpus luteum is caused by $PGF_{2\alpha}$ and oxytocin binding to the endometrium further reinforces $PGF_{2\alpha}$ release so that the two hormones undergo a positive feedback interaction leading to complete luteolysis.

Embryonic Luteotropins

Maintenance of pregnancy requires extension of the life span and function of the corpus luteum. In the ewe it is necessary for the conceptus to be in the uterus by day 12 or 13 postestrus to exert its antiluteolytic effect (Moor and Rowson, 1966). This period is often referred to as the critical period for maternal recognition of pregnancy. Because attachment of the trophoblast to the endometrium in this species does not occur until day 18 postestrus (Amoroso, 1951), the embryo somehow prevents luteolysis 4 to 5 days prior to

attachment to the uterus (Niswender et al., 1985).

The primary mechanism by which the conceptus prevents luteolysis is not fully understood. Measurement of $PGF_{2\alpha}$ concentration in uterine venous plasma revealed no difference between pregnant and nonpregnant animals on comparable days postestrus (Wilson et al., 1972; Pexton et al., 1975; Nett et al., 1976; Lewis et al., 1977; Silvia et al., 1984). In addition, exogenous $PGF_{2\alpha}$ was less effective as a luteolysin in pregnant than in nonpregnant and hysterectomized ewes (Inskeep et al., 1975; Mapletoft et al., 1976; Pratt et al., 1977; Nancarrow et al., 1982). Moreover, the ability of a $PGF_{2\alpha}$ analog to cause luteal regression in superovulated pregnant ewes was inversely related to the number of embryos present in the uterus (Nancarrow et al., 1982). Collectively, these data indicate that the conceptus inhibits the $PGF_{2\alpha}$ luteolytic activity rather than suppresses its secretion.

Maintenance of luteal function during early gestation in domestic animals appears to be regulated by conceptus-derived steroids and proteins that function primarily as antiluteolysins (Stormshak et al., 1987). Rate of uterine blood flow (Griess and Anderson, 1970), uterine secretion of PGE_2 (Marcus, 1981, Silvia et al., 1981; LaCroix and Kann, 1982; Silvia et al., 1984) and protein synthesis (Findlay et al., 1981) all increase at the critical time for maternal recognition of pregnancy.

Treatment of ewes with PGE_2 delayed luteal regression in nonpregnant ewes (Pratt et al., 1977; Magness et al., 1981) and

blocked the luteolytic action of $PGF_{2\alpha}$ when the two compounds were administered simultaneously (Henderson et al., 1977; Mapletoft et al., 1977; Reynolds et al., 1981). In addition, natural and estradiol-induced luteal regression are prevented by intrauterine infusion of PGE_2 or PGE_1 in ewes (Pratt et al., 1977; Colcord et al., 1978; Hoyer et al., 1978; Huecksteadt and Weems, 1978) and heifers (Chenault, 1983). Endometrial slices and dissociated cell preparations from pregnant ewes secreted more PGE_2 than similar preparations from nonpregnant ewes (Ellinwood et al., 1979b; Marcus 1981; LaCroix and Kann, 1982) and PGE_2 is also secreted during incubation of ovine (Hyland et al., 1982; LaCroix and Kann, 1982) and bovine (Shemesh et al., 1979; Lewis et al., 1982) blastocysts.

Prostaglandin E_2 could maintain luteal function through direct action on luteal cells by binding to specific receptors and stimulating adenylate cyclase activity and progesterone synthesis (Fletcher and Niswender, 1982) or by antagonizing the luteolytic actions of $PGF_{2\alpha}$ (Fitz et al., 1984).

Unlike PGE_2, homogenates of ovine embryos had no direct stimulatory effect on secretion of progesterone by cultured ovine luteal cells and did not prolong luteal function when infused directly into the uterine vein (Ellinwood et al., 1979a). In contrast these homogenates prolonged luteal function when infused into the uterine lumen (Rowson and Moor, 1967; Martal et al., 1979; Ellinwood et al., 1979a). However, this luteotropic property was destroyed by treatment with heat or proteolytic enzymes (Rowson and

Moor, 1967; Martal et al., 1979) suggesting that the factor secreted by the embryo, which ultimately resulted in maintenance of the corpus luteum, was a protein.

Characterization of proteins secreted by day 13 ovine conceptus revealed three closely related proteins that are now collectively called ovine trophoblast protein 1 (oTP-1; Godkin et al., 1982). Secretion of oTP-1 occurred transiently between days 12-21 of gestation and appeared to originate from the trophoblast. Bovine conceptuses have also been shown to secrete a group of acidic proteins referred to as bovine trophoblast protein 1 (bTP-1) between days 16-24 of gestation (Bartol et al., 1985). An additional group of bovine conceptus proteins are also produced between days 21-38 of gestation (Godkin and McGrew, 1986).

Infusion of total conceptus proteins, into the uterine lumen of nonpregnant ewes between days 12-18 postestrus (Rowson and Moor, 1967; Martal et al., 1979) and introduction of bTP-1 into the uterine lumen of nonpregnant cows (Thatcher et al., 1985) prolonged the estrous cycle. In addition, ability of trophoblastic vesicles to prolong luteal maintenance after interspecies transfer to recipient cows and ewes, has led to the elegant demonstration of the immunological homology that exists between oTP-1 and bTP-1 (Helmer et al., 1987). Further characterizaion of these conceptus proteins will likely enhance our understanding of their underlying mechanism of action in promoting luteal maintenance during early gestation in ewes and cows.

Paracrine Regulation of Luteal Function

Until recently, central dogma attributed maintenance of the corpus luteum to LH and its demise to $PGF_{2\alpha}$. However, there is increasing evidence that luteal function in domestic animals is controlled not only by secretions from the adenohypophysis and the uterus, but also by secretions from within the ovary. Evidence for paracrine regulation of luteal function is strengthened by the fact that the corpus luteum of several species is composed of two different cell types (Mossman and Duke, 1973); one of which is devoid of LH receptors and does not respond to this hormone, while the other is unresponsive to $PGF_{2\alpha}$ because it lacks receptors for this compound (Niswender et al., 1985). In addition one type of cell secretes a variety of protein and peptide hormones that may affect the metabolism of the other cell type and the function of the corpus luteum in general. Current concepts on the paracrine regulation of luteal function will be reviewed in this section.

Cell Types of the Corpus Luteum

As early as 1919, Corner reported that the porcine corpus luteum was composed of two cell types. Significance of this interesting, yet simple observation was not recognized until recently when it was discovered that porcine (Lemon and Loir, 1977), bovine (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Alila and Hansel, 1984), ovine (Rodgers and O'Shea, 1982; Fitz et al., 1982; O'Shea et al., 1986), rabbit (Hoyer et al., 1986) and primate (Hild-Patito et al., 1986)

corpora lutea consist of small (12-22 um) and large (22-40 um) cells that are morphologically and biochemically different. The small cell is characterized by a spindle, elongated shape, dark-staining cytoplasm, and an irregularly-shaped nucleus. In contrast the basal lamina surrounding large luteal cells is more prominent than that associated with small cells (O'Shea et al., 1979) and the surface of large luteal cells is characterized by the presence of numerous microvillous folds (Enders, 1973). Small and large cells share fine structural features typical of steroid producing cells, namely, numerous mitochondria, abundance of smooth endoplasmic reticulum, and large lipid droplets. In contrast only the large luteal cells possess the cytoplasmic machinery characteristic of cells specialized for polypeptide and protein secretion. This includes numerous Golgi complexes, extensive rough endoplasmic reticulum, and electron dense secretory granules (Niswender et al., 1985).

In addition to these morphological and functional differences the small and large cells differ in several biochemical parameters. There are numerous receptors for LH on small luteal cells but very few on large cells. Conversely, the large cells contain receptors for $PGF_{2\alpha}$ and PGE_2, whereas the small cells do not (Fitz et al., 1982). Moreover, estradiol receptors are fivefold more abundant in large luteal cells (Glass et al., 1985).

Regulation of steroidogenesis in the two cell types appears to be quite different. In particular, the mechanisms involved in regulating steroid secretion by large cells are not clear. The

observation that LH stimulates secretion of progesterone in small cells, but has no effect on large cells is particularly important because large cells secrete approximately 20 times as much progesterone as do small cells in the unstimulated state. Large cells also account for approximately 30% of the corpus luteum on a volume basis compared with 16% for small cells (Niswender et al., 1985). This becomes puzzling if LH is considered to be the luteotropic hormone. However, LH may stimulate the transformation of the small cells into large cells (Donaldson and Hansel, 1965a). This possibility is supported by the recent data of Alila and Hansel (1984) and Niswender et al. (1985). Using monoclonal antibodies developed against theca and granulosa-specific antigens, Alila and Hansel (1984) examined the origin of small and large luteal cells of the bovine corpus luteum during the estrous cycle and gestation. Early in the cycle, on days 4-6, 70% of small cells bound theca antibody, while 77% of large cells bound granulosa antibody. As the cycle progressed, on days 16-18, the number of large cells that bound granulosa antibody decreased to only 30% while 40% of large cells bound theca antibody. After day 100 of gestation, no large cell bound granulosa antibody. These data indicate a gradual increase in the percentage contribution of small cells derived from theca origin to the large cell population as the corpus luteum ages. Niswender et al. (1985) treated ewes with human chorionic gonadotropin (hCG) during midcycle and reported an increase in the number of large cells. If LH transforms small cells into large cells, then it may be

possible that the machinery for progesterone synthesis is "locked on" indefinitely during this transformation process. Only if this is the case could LH be the stimulus for progesterone synthesis by the large cells.

Large luteal cells contain receptors for $PGF_{2\alpha}$ and PGE_2 whereas small cells do not (Fitz et al., 1982). Therefore the luteolytic effects of $PGF_{2\alpha}$ on both large and small cells appear to be mediated by a cytotoxic or inhibitory factor(s) secreted by large luteal cells (Niswender et al., 1985). This may provide another example for paracrine interaction between the large and small cells. The large luteal cells are also stimulated by PGE_2, PGE_1 and prostacyclin to secrete progesterone *in vitro* (Fitz et al., 1984). This suggests that some prostanoids are luteotropic while others are luteolytic. The balance between these chemical species is essential for regulation of luteal function (Khan-Dawood and Dawood, 1986).

Large cells may be more sensitive to estradiol because its receptors are fivefold more abundant than that of small cells (Glass et al., 1985).

Although both small and large cells are steroidogenic, only large cells secrete a variety of protein and peptide hormones. Relaxin (Anderson and Long, 1978; Fields et al., 1980; Fields, 1984), oxytocin (Wathes et al., 1983a; Rodgers et al., 1983; Rice and Thorburn, 1985; Fields and Fields, 1986; Khan-Dawood, 1986) and vasopressin (Wathes et al., 1983a) have been identified in the corpora lutea of domestic animals and primates. Among the peptide

hormones produced by the large luteal cell, oxytocin has received the most attention. Oxytocin concentrations increase in luteal tissue and in the general circulation during the luteal phase of the estrous cycle (Wathes et al., 1986). At low concentrations, oxytocin enhanced basal steroidogenesis in bovine and human cultured luteal cells, while at higher concentrations it inhibited progesterone production and the response of these cells to hCG (Tan et al., 1982a,b). Similarly, oxytocin inhibited LH-stimulated progesterone secretion from cultured small ovine luteal cells indicating that small cells may have oxytocin receptors (Niswender et al., 1985). These observations suggest that oxytocin may serve as an intraluteal communicator between large and small cells.

Aten et al. (1986a,b) reported the presence of a GnRH-like ovarian hormone (GLOH) in rat and human ovaries and proposed that it may be the physiological ligand that binds to the "GnRH" receptors in the ovary because it showed substantial activity in a GnRH radioreceptor assay. Thus this ovarian protein may play a paracrine role by inhibiting luteal function if its effects on the ovary would resemble those of GnRH (see GnRh section).

Extracts of the sheep, goat, pig, dog, cat, rat and human corpus luteum have been shown to contain a substance that inhibits LH binding to its receptor and is referred to as LH-receptor binding inhibitor (LH-RBI; Kumari et al., 1980; Ward , 1981; Yang et al., 1981). The LH-RBI concentration increases in the corpus luteum as it ages. This suggests that LH-RBI may regulate luteal function locally

by inducing luteolysis through inhibition of LH/hCG binding and therefore progesterone biosynthesis in the late luteal phase.

Mechanisms controlling luteal function may involve factors that are produced both within the corpus luteum and outside the ovary. These mechanisms involve a series of molecular species, proteins, peptides, steroids and prostaglandins, each of which may act independently or in concert modifying the actions of one another.

Synthesis and Secretion of Peptide Hormones by the Corpus Luteum

The corpus luteum synthesizes and secretes a variety of protein and peptide hormones. Relaxin (Anderson and Long, 1978; Fields et al., 1980; Fields, 1984), oxytocin (Wathes et al., 1983a; Rodgers et al., 1983; Rice and Thorburn, 1985; Fields and Fields, 1986; Khan-Dawood, 1986), GnRH-like ovarian hormone (GLOH; Aten et al. 1986a,b) and vasopressin (Wathes et al., 1983a) have been identified in the electron-dense secretory granules of the large luteal cells of domestic animals and primates. Synthesis and secretion of oxytocin, GLOH and relaxin from the corpus luteum as well as their actions will be discussed.

Oxytocin in The Corpus Luteum

A corpus luteum factor with oxytocic action was suggested as early as the beginning of this century. Ott and Scott (1910) found that an aqueous extract of corpus luteum increased milk flow in the

goat, and Schafer and Mackenzie (1911) and Mackenzie (1911) showed that injection of an extract of ovine corpus luteum induced milk let-down in the lactating cat. These interesting observations were not pursued further until this decade when oxytocin was identified in corpora lutea of several species. The corpus luteum of the cow (Fields et al., 1983; Wathes et al., 1983a,b) ewe (Wathes and Swann, 1982) monkey (Khan-Dawood et al., 1984) woman (Wathes et al., 1982; Khan-Dawood and Dawood, 1983) and rabbit (Khan-Dawood and Dawood, 1984) has been shown to contain measurable quantities of oxytocin.

Comparison of Luteal and Hypothalamic Oxytocin

Luteal oxytocin is not only immunologically indistinguishable, but is also biologically similar to its pituitary counterpart. Luteal extracts caused a significant increase in contraction of uterine muscle, stimulated contraction of uterine strips *in vitro* and increased intramammary pressure in a manner similar to authentic oxytocin in rats (Wathes and Swann, 1982; Wathes et al., 1983b). Moreover ovine, bovine and human luteal oxytocin extracts elute at the same position as pituitary oxytocin by Sephadex G50 and reverse-phase high-performance liquid chromatography (HPLC; Wathes and Swann, 1982; Wathes et al., 1982; Wathes et al., 1983a; Fields et al., 1983; Sheldrick and Flint, 1983a; Schaeffer et al., 1984; Dawood and Khan-Dawood, 1986). Dispersed cell cultures of ovine and bovine corpora lutea incorporated labeled cysteine into a peptide that eluted at the

same position as oxytocin on HPLC. As occurs in the hypothalamus, the synthesis of luteal oxytocin involved the formation of an approximately 14-K precursor protein that was subsequently cleaved to form oxytocin and neurophysin (Swann et al.,1984). The oxytocin gene is highly transcribed in the bovine corpus luteum. Luteal cDNA sequence analysis as well as cell-free translation studies showed that luteal and hypothalamic mRNA for oxytocin were essentially similar. However, the active corpus luteum produces approximately 250 times more oxytocin mRNA than a single hypothalamus (Ivell and Richter, 1984).

Variations in Luteal Oxytocin Levels

In the ewe and cow oxytocin is first detectable in follicular fluid and granulosa cells during or shortly after the LH surge (Kruip et al., 1985; Wathes et al., 1986). Measurement of luteal oxytocin-specific mRNA throughout the estrous cycle of the cow showed that gene transcription was maximal accompanying ovulation and decreased thereafter. Maximal concentrations of mRNA were detected around day 3; these declined sharply around day 7 and reached a basal level by day 11 of the cycle, after which only very low levels were detectable (Ivell et al., 1985). Measurement of oxytocin mRNA levels in the bovine hypothalamus revealed no significant variation due to stage of the estrous cycle (Ivell et al., 1985). This implies that the factors that regulate transcription of the oxytocin gene are tissue-

specific and that the hypothalamic gene is regulated independently from its luteal counterpart (Ivell, 1986).

Changes in luteal concentrations of oxytocin during the bovine estrous cycle apparently do not occur simultaneously with those of mRNA for this peptide. Oxytocin concentrations in ovine and bovine luteal tissues have been shown to be maximal at day 8 and between days 5-10, respectively (Sheldrick and Flint, 1983b; Wathes et al., 1984). In the cow, luteal oxytocin concentrations increased from about .5 μg/g (wet weight) on days 1-4 of the cycle to more than 1.7 μg/g on days 5-10 and declined thereafter to about 1 μg/g between days 11-17 and to less than .6 μg/g after day 18 (Wathes et al., 1984). A similar pattern was observed for the ewe by Sheldrick and Flint (1983b) who found maximal oxytocin concentration in ovine luteal tissue on day 8 of the cycle. The observed difference in time between occurrence of maximal levels of oxytocin mRNA and of concentrations of this peptide hormone in luteal tissue suggests that during early stages of the cycle only some of the prohormone is processed. This lag period may be controlled by certain endocrine factors that regulate synthesis and processing of the hormone in granulosa cells (Wathes et al., 1986).

In contrast to the high oxytocin concentrations measured in ruminants, extremely low concentations were found in the sow corpus luteum, with maximal levels (10 ng/g) detected on day 5 of the cycle (Pitzel et al., 1984). Similarly, both rat and rabbit ovarian and

luteal tissues, respectively, contained low levels of oxytocin (Khan-Dawood and Dawood, 1984). Oxytocin concentration in primate luteal tissue was also much lower than that of ruminants. Wathes et al. (1982) detected oxytocin levels of about 30 ng/g while Khan-Dawood and Dawood (1983) reported oxytocin concentrations of 59 ng/g in human luteal tissue. In cynomolgus monkeys the concentration ranged from 34 to 602 ng/g wet weight of tissue, with the highest concentration at the midluteal phase of the cycle (Khan-Dawood et al., 1984).

Oxytocin is produced by the large luteal cells and is temporarily stored in membrane-bounded secretory granules (Rodgers et al., 1983; Rice and Thorburn, 1985; Kruip et al., 1985; Fields and Fields, 1986). This peptide hormone is secreted into ovarian veins of cows (Walters et al., 1984) and ewes (Flint and Sheldrick, 1982), thereby contributing to the relatively high levels of oxytocin in the systemic circulation of these species during the luteal phase of the estrous cycle (Webb et al., 1981; Sheldrick and Flint, 1981; Mitchell et al., 1982; Schams et al., 1982; Schams, 1983; Walters et al., 1984; Walters and Schallenberger, 1984). In contrast, the follicular phase in both species is characterized by low circulating oxytocin concentrations that decline to basal levels at the time of estrus (Wathes et al., 1986). This decline in oxytocin concentrations either preceded or occurred simultaneously with that of progesterone (Schams, 1983; Flint and Sheldrick, 1983).

Control of Luteal Oxytocin Secretion

As discussed in a previous section of this review, ovarian secretion of oxytocin is believed to be stimulated by $PGF_{2\alpha}$. Indeed, treatment of cows and ewes with an analog of $PGF_{2\alpha}$ caused an immediate increase in luteal oxytocin secretion (Walters et al., 1983; Flint and Sheldrick, 1983; Schallenberger et al., 1984) and degranulation of luteal cells (Heath et al., 1983). Evidence for endogenous $PGF_{2\alpha}$-stimulated release of ovarian oxytocin is provided in part by the close relationship that exists between the occurrence and frequency of episodic secretion of this ovarian hormone and that of 13,14-dihydro-15-keto-$PGF_{2\alpha}$, a stable metabolite of $PGF_{2\alpha}$ (Flint and Sheldrick, 1982). Conversely, oxytocin treatment stimulated the release of $PGF_{2\alpha}$ from the uterine endometrium of the ewe (Roberts and McCracken, 1976), goat, (Cooke and Homeida, 1982) and cow (Newcomb et al., 1979; Milvae and Hansel, 1980).

Actions of Ovarian Oxytocin

The first evidence of a luteolytic effect of oxytocin was described by Armstrong and Hansel (1959) and Hansel and Wagner (1960). Injection of oxytocin into heifers between days 3-6 of the cycle resulted in a significant decrease in duration of estrous cycle. These data were later confirmed in the cow (Labhsetwar et al., 1964; Donaldson and Takken, 1968; Harms et al., 1969) and goat (Cooke and Knifton, 1981; Cooke and Homeida, 1982). It appears that

the luteolytic action of oxytocin is normally mediated by $PGF_{2\alpha}$ as explained above because it can be prevented by hysterectomy in the cow (Armstrong and Hansel, 1959; Anderson et al., 1965; Ginther et al., 1967) and by simultaneous inhibition of prostaglandin synthesis in the goat (Cooke and Knifton, 1981; Cooke and Homeida, 1983). Further, both active and passive immunization against oxytocin extended the length of the cycle in ewes and goats (Sheldrick et al., 1980; Schams et al., 1983; Cooke and Homedia, 1985). In the ewe, rhesus monkey, mare, sow, rat, rabbit and guinea pig injection of oxytocin has thus far proved ineffective in reducing estrous cycle length (Duncan et al., 1961; Donovan, 1961; Brinkley and Nalbandov, 1963; Milne, 1963; Neely et al., 1979; Wilks, 1983).

Oxytocin may also have a direct effect on steroidogenesis and regulation of luteal function. In low concentrations, oxytocin stimulated progesterone production by isolated bovine and human luteal cells in the early luteal phase, whereas, it inhibited both basal and hCG-stimulated progesterone release at high concentrations (Tan et al., 1982a,b). This latter effect of oxytocin on progesterone production was confirmed by Niswender et al. (1985) who found that oxytocin inhibited the response of small ovine luteal cells to hCG *in vitro*. However, numerous studies conducted by others have failed to confirm that oxytocin can act directly at the level of the corpus luteum in human (Richardson and Masson, 1985), rat (Mukhopadhyay et al., 1984) and cow (Wathes et al., 1986) to suppress

progesterone secretion. Nevertheless, Adashi and Hsueh (1981a,b), using rat testicular cells in culture, have demonstrated a dose-dependent inhibitory effect of oxytocin, vasopressin and vasotocin on testosterone secretion. It was later shown that the vasotocin effect was due to inhibition of the enzymes 17α-hydroxylase and 17-20 desmolase that convert progesterone to androstenedione (Adashi and Hsueh, 1982). A similar site of action of oxytocin in the corpus luteum is likely to inhibit androgen and estrogen rather than progesterone production by the ovary (Wathes et al., 1986).

A role for oxytocin in regulating steroidogenesis is supported by the recent findings that oxytocin is also produced in other steroidogenic tissues such as the adrenal cortex, placenta, and testis (Fields et al., 1983; Makino et al., 1983; Nicholson et al., 1984; Schams et al., 1985a). Concentration of placental oxytocin increased during the second trimester and remained high until term (Lederis and Jayasena, 1970). In the testis, immunocytochemical studies have shown that 80% of interstitial cells stain for oxytocin. This suggests that oxytocin is produced by the Leydig cells (Guldenaar and Pickering, 1985). Oxytocin in both placenta and testis may stimulate uterine and tubular motility, respectively (Wathes et al., 1986).

GnRH-Like Ovarian Hormone

Gonadotropin releasing hormone (GnRH) of hypothalamic origin was

thought to act exclusively on the pituitary gland to increase release of LH and FSH. However an extrapituitary action of this releasing hormone was suggested by Rippel and Johnson (1976) who demonstrated the inhibitory effect of a GnRH-agonist on hCG-stimulated augmentation of ovarian and uterine weights in immature hypophysectomized rats. Since this initial observation, inhibitory effects of GnRH or its agonistic analogs on luteal function has been demonstrated in many species including the rat (Kledzik et al., 1978; Harwood et al., 1980; Jones and Hsueh, 1980), human (Koyama et al., 1978; Casper and Yen, 1979), monkey (Asch et al., 1981) and cow (Rodger and Stormshak, 1986).

Exogenous GnRH or GnRH-agonistic analogs may act directly on the ovary to alter steroidogenesis. Treatment of primary cultures of rat granulosa cells with GnRH or its agonists inhibited FSH-stimulated estrogen and progestin production (Hsueh and Erickson, 1979; Hsueh and Ling, 1979), and FSH-stimulated LH and prolactin (PRL) receptor formation (Hsueh and Ling, 1979; Hsueh et al., 1980). These inhibitory effects of GnRH on granulosa cells were blocked by concomitant treatment with a GnRH antagonist (Hsueh and Ling, 1979; Hsueh et al., 1980). In addition to suppression of steroidogenesis and receptor formation, GnRH has been shown to inhibit FSH-stimulated cAMP formation (Clark et al., 1980; Knecht et al., 1981), and enhance FSH-stimulated prostaglandin production by rat granulosa cells (Clark et al., 1980). Treatment with GnRH also inhibited progesterone

production stimulated by hCG, epinephrine, or LH during short term incubation of rat luteal cells (Clayton et al., 1979; Harwood et al., 1980; Behrman et al., 1980; Massicotte et al., 1981). However, higher levels of LH and hCG alleviated the inhibitory effects of GnRH on progesterone production in the rat (Behrman et al., 1980) and human (Casper et al., 1980), respectively.

Although the rat model has been used extensively to examine the effects of GnRH on ovarian functions, direct effects of GnRH are not limited to this species. In vitro treatment with GnRH or its agonists modulates steroidogenesis in ovarian cells from pigs (Massicotte et al., 1981) and chickens (Takats and Hertelendy, 1982; Hertelendy et al., 1982). In contrast no direct inhibition of steroidogenesis by GnRH was found in studies using ovarian tissues of mice and rhesus monkeys (Asch et al., 1981; Bex et al., 1982)

The bovine corpus luteum may be more sensitive to GnRH treatment during the mid than early luteal phase of the cycle. A single intravenous injection of GnRH into cows on day 2 of the cycle was followed by a 6 day lag period before altered luteal function was detected. On the other hand, a similar injection on day 10 of the cycle caused serum concentrations of progesterone to be significantly depressed after only 48 h (Rodger and Stormshak, 1986). Other investigators have shown that injection of GnRH or a GnRH agonistic analog into cows during the midluteal phase of the cycle increased serum concentrations of progesterone (Kittok et al., 1973; Milvae et

al., 1984). However, Milvae et al. (1984) reported that luteal function of GnRH-treated cows was suppressed during the succeeding cycle.

Several attempts have been made to elucidate the mechanism(s) by which GnRH affects ovarian function. Casper et al. (1980) suggested that GnRH-induced early luteal regression in the human may be due to a decrease in gonadotropin secretion caused by GnRH-induced pituitary refractoriness. In the cow GnRH injection caused release of LH that may have resulted in down-regulation of luteal LH receptors (Rodger and Stormshak, 1986). It has also been proposed that the inhibitory action of GnRH in rat luteal cells results from ability of the decapeptide to inhibit calcium extrusion from the cytosol (Williams and Behrman, 1983). Consequently, elevated levels of intracellular calcium prevent activation of adenylate cyclase by LH via a protein kinase C-dependent phosphorylation or calmodulin-dependent process. This mechanism of action of GnRH is supported by recent data of Leung et al. (1986) who found that GnRH binding to receptors in plasma membranes of rat luteal cells in primary culture activated hydrolysis of phosphoinositides, which ultimately leads to stimulation of protein kinase C activity.

To demonstrate the physiological significance of the direct ovarian actions of GnRH, it was deemed necessary to establish the presence of a GnRH-like substance as well as its receptors in the ovary. The presence of such a molecule would explain the paradoxical

inhibitory effect of this releasing peptide on ovarian functions and provide unique models for understanding the mechanism of GnRH action on steroidogenesis and ovulation (Hsueh et al., 1984). Gonadotropin releasing hormone receptors have been detected in rat luteal cells (Clayton et al., 1979), but only low affinity binding sites have been found in the human corpus luteum (Clayton and Huhtaniemi, 1982; Popkin et al., 1983). However, no GnRH receptors have been detected in bovine, ovine and porcine luteal tissue (Brown and Reeves, 1983). Although several reports have suggested that GnRH or a GnRH-like peptide is synthesized in the ovary and exerts its effects locally (Williams and Behrman, 1983; Birnbaumer et al., 1985), only recently has the presence of such a molecule been demonstrated in both human and rat luteal tissues (Aten et al., 1986a,b). Rat ovarian extract was found to contain a GnRH-like protein that exibited the same membrane binding properties as GnRH but was distinctly different from the hypothalamic decapeptide in several respects. Although this ovarian protein showed substantial activity in the GnRH radioreceptor assay, it was immunologically refractory to a sensitive and specific GnRH antiserum and exhibited only little immunoassayable activity. Although, both radioreceptor and immunological activities were detected in the hypothalamus, both activities were absent from plasma extracts of the same animals. In addition, the GnRH-like activity of the ovarian extracts was sensitive to elevated temperatures that did not affect its hypothalamic counterpart. Gonadotropin releasing

hormone-like activity was significantly reduced by incubation at either 50 or 60°C for as little as 5 min, but GnRH was not affected by incubation at 60°C for up to 60 min. Moreover, the GnRH-like activity of ovarian extracts did not behave like GnRH when fractionated by reverse phase HPLC. The peaks did not elute with authentic GnRH when ovarian extracts containing GnRH were fractionated by an identical procedure (Aten et al., 1986b). In a subsequent experiment Aten et al. (1986a) reported the presence of a GnRH-like ovarian hormone (GLOH) in the human ovary. This molecule was similar if not identical to GLOH of the rat. They proposed that GLOH may be the physiological ligand that binds to the so-called GnRH receptors in the rat ovary and speculated that receptors for GLOH may be present in human ovaries as well. Knowledge of the significance of GLOH in control of ovarian function in rat, human and other species awaits the purification of this protein.

Relaxin in the Corpus luteum

Evidence for a unique principle in aqueous extracts of swine corpora lutea that relaxed the guinea pig pubic symphysis was first presented by Hisaw (1926, 1927). Since then relaxin has been identified in ovaries of the pig (Sherwood and O'Byrne, 1974; Matsumoto and Chamley, 1980; Fields et al., 1982), rat (Niall et al., 1982), cow (Fields et al., 1980; Fields et al., 1982), human (Weiss et al., 1976; O'Byrne et al., 1978) and monkey (Weiss et al., 1981).

Relaxins are species specific peptides with molecular weights of just under 6000 daltons. They are composed of two nonidentical peptide chains, A and B, covalently linked by two disulfide bridges with an additional intrachain disulfide bridge in the smaller A chain (John et al., 1981). Relaxin is derived from a larger precursor in which A and B chains are linked by a connecting C peptide (Niall et al., 1982). The hormone resembles insulin in size, tertiary structure and mechanisms of cleavage from the primary RNA transcripts (Schwabe et al., 1978). This has led to the speculation that relaxin and insulin are derived from an ancient gene that underwent duplication and modification. However, this concept has been recently challenged on the basis of very limited homology in amino acid sequence between the two hormones (Schwabe et al., 1982).

Although relaxin is produced during the estrous cycle in low concentrations, the primary source of this hormone is the corpus luteum of pregnancy (Anderson et al., 1982). Wada and Yuhara (1961) suggested that relaxin is present in the cow after observing that plasma from pregnant cows provoked a positive response in the guinea pig bioassay for relaxin. Relaxin was isolated from bovine corpora lutea during late pregnancy by procedures including acid-acetone extraction, gel filtration, and isoelectric focusing, and its biological activity was determined by the assay of mouse uterine-motility inhibition and by the mouse interpubic ligament bioassay. Three major active fractions were obtained with relaxin activity

chromatographed in the ranges of 1400 and 6,000 daltons (Fields et al., 1980). However, isolation and purification of relaxin from bovine corpora lutea were complicated by the low concentrations of relaxin and presence of a factor that increased contraction of the uterus in the mouse uterus bioassay that led to inconsistent measures of relaxin biological activity (Fields et al., 1980). This factor was later determined to be oxytocin (see oxytocin section).

Unlike bovine luteal tissue, its porcine counterpart is characterized by low oxytocin concentrations and higher relaxin levels. Therefore the pig is the best studied model for relaxin. In sows and gilts relaxin was first detected in follicular fluid, which contains a wide range of concentrations (Matsumoto and Chamley, 1980). Levels of this hormone in sow luteal tissue remain low throughout the estrous cycle. In pregnant sows, relaxin is produced by granulosa lutein cells and stored in electron-dense secretory granules. Number of relaxin containing-granules increases steadily throughout pregnancy to become maximal by days 105-110 of gestation. By the day preceding delivery, the number of these granules begins to decline and within 6 h of parturition all granulosa lutein cells are nearly devoid of these granules. (Anderson et al., 1982). This pattern agrees very well with relaxin secretion from the ovary. Systemic plasma concentrations of porcine relaxin remain low during the first 100 days of gestation, increase gradually within 3 days before delivery and rise during the next 2 days to peak

concentrations 22 h before parturition (Sherwood et al., 1975).

When relaxin was first discovered, the impression was gained that it functioned primarily to prepare the birth canal for passage of term fetuses. Exogenous relaxin elicited marked dilatation in the uterine cervix in ovariectomized heifers and sows pretreated with diethylstilbestrol (Zarrow et al., 1956; Smith and Nalbandov, 1958). This was later confirmed by Anderson et al. (1982) who found exogenous porcine relaxin to induce cervical dilatation at a stage of gestation when endogenous blood levels of estrogens reach peak values and progesterone levels begin to decline. In mice, hamsters and rats, cervical dilatation during the latter one-half of gestation coincides with increasing systemic blood concentrations of immunoreactive relaxin (Steinetz et al., 1980). In addition to cervical softening and dilatation, relaxin induces pubic symphysis relaxation, inhibits myometrial activity and affects the vagina and mammary glands (Schwabe et al., 1978). Relaxin has also been implicated in the separation of the placenta from the uterus and weakening of the fetal membranes (Weiss, 1981), and may act as an insulin-like regulator of carbohydrate and protein metabolism in reproductive tissues. Purified porcine relaxin stimulated glycogen deposition, tissue growth and protein synthesis as indicated by amino acid incorporation in uteri of either unprimed or estrogen-primed ovariectomized rats (Frieden et al., 1982).

STATEMENT OF THE PROBLEM

In order to maximize the reproductive efficiency of domestic animals, efforts have been made to develop effective methods for regulating the life span of the corpus luteum. Oxytocin has been implicated in the regulation of the estrous cycle when it was demonstrated that treatment of cows with this nonapeptide resulted in shorter cycles. It was later demonstrated that oxytocin is produced by large cells of the bovine and ovine corpus luteum and stored in secretory granules, but factors controlling its synthesis and release are poorly understood.

Oxytocin concentrations in bovine luteal tissue at different stages of the estrous cycle are not well defined. However, oxytocin and progesterone secretions appear to change concomitantly during the cycle. Moreover, in vivo ovarian secretion of oxytocin is stimulated by $PGF_{2\alpha}$ in both cows and ewes.

Research presented in this thesis was undertaken to determine oxytocin concentrations in luteal tissue at different stages of the estrous cycle and to examine in vitro effects of $PGF_{2\alpha}$, PGE_2 LH, cycloheximide, colchicine, and cytochalasin B on oxytocin synthesis and release.

EXPERIMENTS 1 AND 2: PROSTAGLANDIN $F_{2\alpha}$-INDUCED RELEASE OF OXYTOCIN FROM BOVINE CORPORA LUTEA IN VITRO

INTRODUCTION

Oxytocin has been identified in bovine (Fields et al., 1983; Wathes et al., 1983a,b) and ovine (Wathes and Swann, 1982) corpora lutea where it is temporarily stored in granular form in large luteal cells (Fields and Fields, 1986). This peptide hormone is secreted into ovarian veins of cows (Walters et al., 1984) and ewes (Flint and Sheldrick, 1982), thereby contributing to relatively high levels of oxytocin in the systemic circulation of these species during the luteal phase of the estrous cycle (Webb et al., 1981; Sheldrick and Flint, 1981; Schams, 1983). Changes in luteal oxytocin concentrations during the bovine estrous cycle are not well defined because available data are based on subjectively dated tissue acquired at the abattoir (Wathes et al., 1984; Schams et al., 1985a).

Ovarian secretion of oxytocin is stimulated by $PGF_{2\alpha}$. Treatment of cows with a $PGF_{2\alpha}$ analog caused an immediate increase in luteal oxytocin secretion (Walters et al., 1983; Schallenberger et al., 1984) and degranulation of luteal cells (Heath et al., 1983). Administration of the same analog to ewes (Flint and Sheldrick, 1983) has also been shown to result in a significant veno-arterial difference in oxytocin levels in ovaries bearing corpora lutea . In addition, a transient increase in systemic plasma oxytocin that

occurred in $PGF_{2\alpha}$-treated intact ewes was absent in similarly treated ovariectomized ewes. Evidence for endogenous $PGF_{2\alpha}$-stimulated release of ovarian oxytocin is provided in part by the close relationship that exists between the occurrence and frequency of episodic secretion of this ovarian hormone and that of 13,14-dihydro-15-keto-$PGF_{2\alpha}$, a stable metabolite of $PGF_{2\alpha}$ (Flint and Sheldrick, 1982).

Results of these studies implicate $PGF_{2\alpha}$ as a major regulator of ovarian oxytocin secretion. However, it has been demonstrated that secretion of neurohypophyseal oxytocin is stimulated by PGE_2 (Negro-Vilar et al., 1985), which is synthesized by the uterus and is known to regulate the life span of the corpus luteum (Silvia et al., 1984). In addition, changes in plasma oxytocin and progesterone concentrations have been found to occur concomitantly during the estrous cycle in cattle and sheep (Sheldrick and Flint, 1981; Schams et al., 1982; Walters et al., 1984). Because LH regulates progesterone synthesis by the bovine corpus luteum it is possible that it may also affect oxytocin secretion. The roles of PGE_2 and LH, if any, in altering secretion of oxytocin have not been investigated.

This study was conducted to determine oxytocin concentrations in bovine corpora lutea at specific stages of the estrous cycle and to examine the <u>in vitro</u> effects of $PGF_{2\alpha}$, PGE_2 and LH on oxytocin release from this tissue at the same stages of the cycle.

MATERIALS AND METHODS

Experiment 1

In vitro effects of $PGF_{2\alpha}$, PGE_2 and LH on oxytocin release from the bovine corpus luteum at different stages of the estrous cycle were investigated. Sixteen Hereford and Hereford x Angus heifers (2 years old; 350-400 kg) were observed twice daily for estrus using vasectomized bulls. After exhibiting at least two consecutive estrous cycles of normal duration (18-23 days), heifers were assigned randomly in equal numbers to be slaughtered on each of days 4, 8, 12, and 16 of the estrous cycle (day of detected estrus = day 0 of the cycle).

Approximately 20 min after slaughter, the ovary bearing the corpus luteum was collected, placed in cold Ham's F-12 medium containing 24 mM Hepes buffer, 100 U/ml penicillin, 100 ug/ml streptomycin, 2.5 ug/ml fungizone and supplemented with 5 ug/ml insulin, 5 ug/ml transferrin and 5 ng/ml selenium (Pate and Condon, 1984) and transported to the laboratory (10 min). The corpus luteum was dissected from the ovarian stroma, weighed and a small portion (500-600 mg wet weight) of tissue was removed and stored at -20°C for determination of the original concentration of oxytocin. The remaining luteal tissue was sliced (0.3 mm thickness), washed four times with 40 ml of Ham's F-12, blotted on a dampened filter paper and subsequently divided into 200-300 mg aliquots. These tissue aliquots were placed into one of four incubation flasks (prepared in duplicate) containing 2.955 ml of Ham's F-12 to which was added the

following: 1) vehicle (control, 30 ul ethanol, 15 ul normal saline), 2) 30 ng $PGF_{2\alpha}$, 3) 30 ng PGE_2 and 4) 15 ng LH. Prostaglandins $F_{2\alpha}$ and E_2 were dissolved in 30 ul of ethanol and LH was dissolved in 15 ul of physiological saline. Flasks to which prostaglandins had been added also received 15 ul physiological saline and those to which LH had been added received 30 ul ethanol. All flasks were flushed with 95% O_2-5% CO_2, stoppered and incubated in a Dubnoff metabolic incubator at 38°C for 2 h. Incubation was terminated by immersing the flasks in ice water (4°C). The contents were then transferred to glass tubes and centrifuged at 3000 x g, after which the tissue slices were separated from the supernatant and both were immediately frozen and stored at -20°C pending extraction and quantification of oxytocin. Tissue from heifers on day 4 was sufficient only for determination of initial oxytocin levels.

Experiment 2

The dose-response effect of $PGF_{2\alpha}$ on *in vitro* oxytocin synthesis and(or) release from the bovine corpus luteum on day 8 of the estrous cycle was investigated. Six heifers similar to those utilized in experiment 1 were slaughtered on day 8 of the estrous cycle. Corpora lutea were collected, and a small sagittal portion of tissue was removed and stored at -20°C for the determination of the original oxytocin concentration . The remaining tissue was sliced, washed and

aliquots of 200-210 mg were placed into five sets of duplicate flasks containing 2.97 ml of Ham's F-12 medium. Flasks and the treatments were as follows: flasks 1 and 2) unincubated and incubated control tissue, respectively; flasks 3, 4, and 5) tissue incubated with 10, 20 and 40 ng $PGF_{2\alpha}$/ml of incubation medium, respectively. Total quantity of $PGF_{2\alpha}$ (30, 60 or 120 ng) to be added to the appropriate flasks was dissolved in 30 ul of absolute ethanol and an equivalent volume of this vehicle was added to each control flask. Incubation of tissue was performed for 2 h as described for experiment 1.

Oxytocin Extraction

Oxytocin in the incubation media was assayed directly without extraction. Tissue oxytocin was extracted by a modification of the method described by Flint and Sheldrick (1983). Tissue samples were homogenized using a glass pestle and tube in 10 ml 1% acetic acid, after addition of approximately 4,000 cpm ($U-^{3}H$)-oxytocin (New England Nuclear, Boston, MA) to allow for calculation of recoveries. Homogenates were centrifuged at 10,000 x g for 30 min at 4°C and the supernatants freeze-dried. Residues were reconstituted in 5 ml assay buffer (0.05 M phosphate buffer, 50 mM EDTA, and 0.5 g/liter gelatin, pH 7.5) and centrifuged at 10,000 x g for 30 min at 4°C after which supernatants were assayed for oxytocin. Mean extraction recovery was 85.0 $\pm$ 5.5% (N = 12 assays).

Oxytocin Radioimmunoassay

Oxytocin was measured by radioimmunoassay according to a modification of the validated method described by Schams (1983). The assay was performed in silicone coated glass tubes (12x75mm), using 0.05 M phosphate buffer with 50 mM EDTA and 0.5 g/liter gelatin, pH 7.5, as diluent. Two hundred microliters of extract or oxytocin standard (450 units/mg; Calbiochem, San Diego, CA) were incubated for 24 h at 4-6°C with 100 ul antiserum (generously donated by Dr. D. Schams, Physiological Institute, Technical University of Munich, West Germany) in a final dilution of 1:100,000. About 5,000 cpm of (^{125}I)iodo-oxytocin (New England Nuclear, Boston, MA) in 100 ul buffer were added to each tube and the incubation was allowed to continue for another 48 h. One hundred microliters of 3% bovine serum albumin were then added, followed by 400 ul dextran-coated charcoal solution (0.66% w/v neutral norit and 0.066% w/v dextran T-70 in buffer). The tubes were then incubated for 20 min and centrifuged at 4°C at 3,000 x g for 15 min. Following centrifugation, 0.5 ml of the supernatant was removed and counted. Specificity of oxytocin antiserum used in this study was previously validated (Schams et al., 1979; Schams, 1983). Mean recovery of various amounts of standard oxytocin (0.25-24.0 pg) added to pooled cow plasma extract as validated in our laboratory was 98.2 $\pm$ 6.2%. Sensitivity of the assay was 0.25 pg/tube ($P<0.05$, N = 32). Intra and interassay coefficients of variation were 3.6 and 9.6%,

respectively.

Statistical Analyses

Statistical analyses were performed according to Snedecor and Cochran (1980). Data from experiment 1 were analyzed by one and two-way analyses of variance. Data from experiment 2 were analyzed by one-way analysis of variance and differences among groups were assessed by orthogonal contrasts. Linearity of the dose-response curve was tested for significance by computing the F values for the linear and quadratic components of the sums of squares for dose.

RESULTS

Experiment 1

Luteal concentration of oxytocin varied markedly throughout the estrous cycle ($P<0.05$, Table 1) increasing from day 4 to 8 and thereafter declining through day 12 to lowest levels on day 16.

In vitro release of oxytocin from luteal tissue differed among stages of the cycle studied ($P<0.01$). In parallel with the initial concentration of oxytocin present in the tissue, *in vitro* release of this hormone in response to incubation alone was greatest on day 8 compared with day 12 and lowest on day 16 (Fig. 1). Prostaglandin E_2 and LH did not differ in their ability to stimulate release of oxytocin at each stage of the cycle nor did the quantity of oxytocin released in their presence differ from that released in their

TABLE 1. Oxytocin concentrations (mean ± SE) in bovine luteal tissue at different stages of the estrous cycle

Day of estrous cycle	No. of animals	Oxytocin ($ng \cdot g^{-1} \cdot CL^{-1}$)
4	4	414 ± 84^{a}
8	4	2019 ± 330^{b}
12	4	589 ± 101^{c}
16	4	81 ± 5^{d}

^{a,b,c,d}Means with a different superscript letter differ ($P<0.05$).

absence. However, relative to the control, $PGF_{2\alpha}$ increased ($P<0.05$) the release of oxytocin on day 8 of the cycle but not on days 12 or 16. Luteal tissue on day 8 of the cycle not only released greater quantities of oxytocin in response to $PGF_{2\alpha}$ but after incubation was also found to contain more oxytocin than that of control tissue ($P<0.05$; Fig. 2). It is conceivable that this was a chance observation based on the limited sample size because in the subsequent experiment a similar response to $PGF_{2\alpha}$ was not detected. Nevertheless, total oxytocin levels (tissue + medium) were consequently greater in flasks to which $PGF_{2\alpha}$ was added than that of control flasks ($P<0.05$; Fig. 2). A similar response to $PGF_{2\alpha}$ on days 12 and 16 was not detected, with total oxytocin being comparable to that of respective controls.

Experiment 2

Response of luteal tissue to increasing concentrations of $PGF_{2\alpha}$ is depicted in Table 2. Incubation of luteal tissue in the presence of increasing concentrations of $PGF_{2\alpha}$ caused a significant linear increase ($P<0.05$) in the quantity of oxytocin released into the medium. By analysis of variance the linear but not the quadratic component of the sums of squares for dose was found to be significant. Surprisingly, oxytocin concentration (ng/g) of unincubated sliced tissue (2692 ± 159) was significantly greater than that of nonsliced tissue (1658 ± 42) suggesting that some synthesis

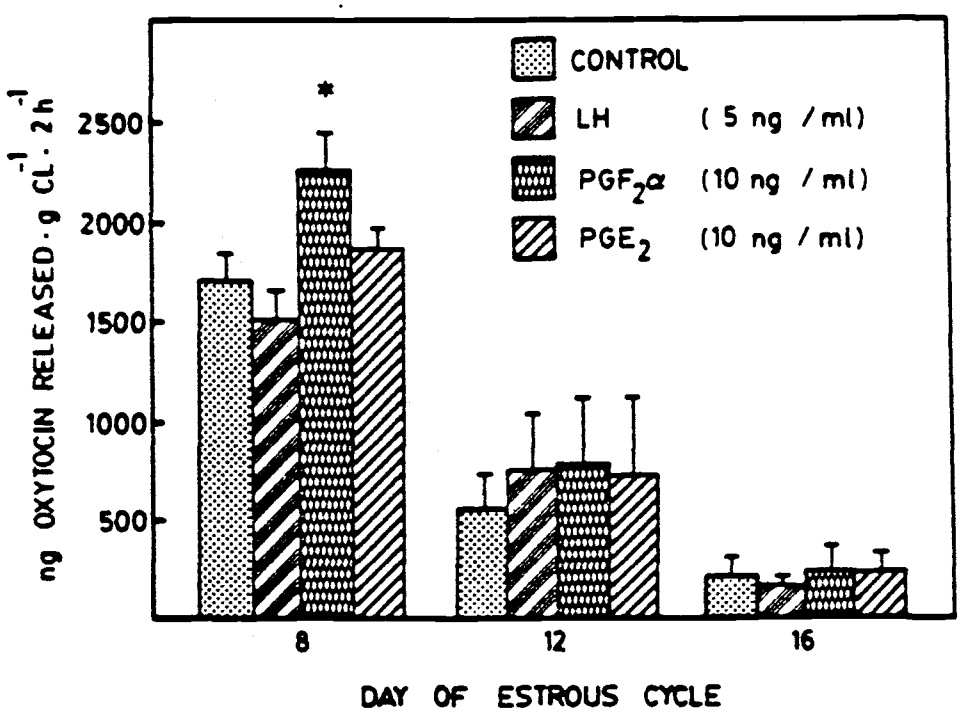

Fig. 1. Oxytocin released (mean ± SE) into medium after 2 h incubation of luteal tissue with LH, $PGF_{2\alpha}$ or PGE_2 on days 8, 12 and 16 of the estrous cycle. Responses to treatments differed among stages of the estrous cycle ($P<0.01$).

* Different from control ($P<0.05$).

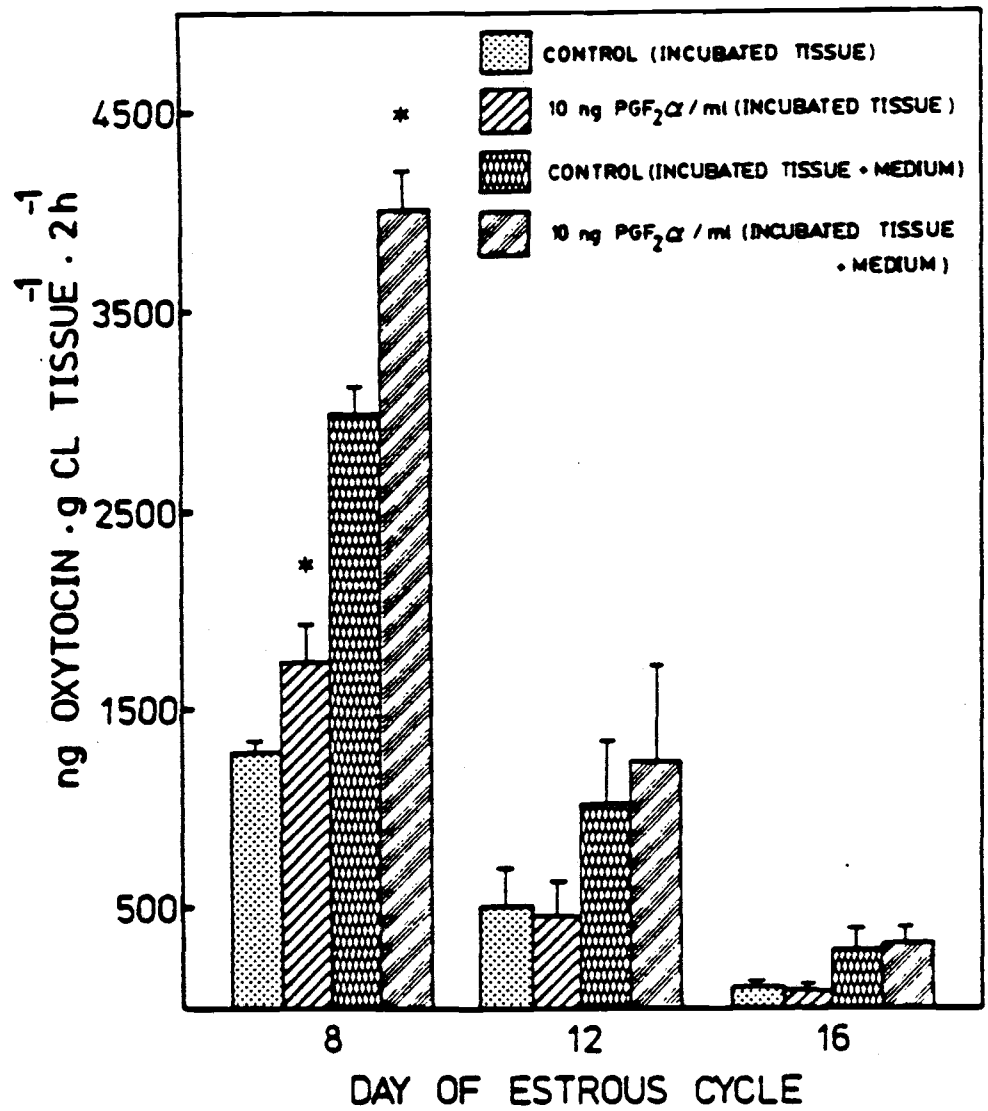

Fig. 2. Concentrations of oxytocin (mean ± SE) in luteal tissue slices and in tissue + medium after 2 h incubation with $PGF_{2\alpha}$ on days 8, 12 and 16 of the estrous cycle.

* Different from respective controls ($P<0.05$).

TABLE 2. Oxytocin synthesis and(or) release by luteal tissue in response to various levels of $PGF_{2\alpha}$ *in vitro*

Treatment*	Mean oxytocin concentration ($ng \cdot g^{-1} \cdot 2h^{-1}$)		
	Incubation medium	Tissue	Tissue + medium
Control	1824[a]	1720[a]	3498[a]
10 ng $PGF_{2\alpha}$/ml	2116[b]	1889[a]	3943[b]
20 ng $PGF_{2\alpha}$/ml	2332[c]	1906[a]	4163[b]
40 ng $PGF_{2\alpha}$/ml	2436[c]	1952[a]	4312[b]
Common SE	66	157	150

*Luteal tissue obtained from six heifers on day 8 of the estrous cycle was incubated with various levels of $PGF_{2\alpha}$ for 2 h at 38°C.

[a,b,c]Means within a column with a different superscript letter differ ($P<0.05$).

and(or) processing of the prohormone occurred during handling in preparation for incubation. Oxytocin concentration in luteal tissue after incubation did not differ among treatments but total concentration of oxytocin (tissue + medium) was different ($P<0.05$; Table 2).

DISCUSSION

In this study luteal oxytocin levels increased from day 4 to 8 of the estrous cycle and then declined through the remainder of the cycle. These data are in agreement with those of others (Schams, 1983; Wathes et al., 1984) who found the highest concentration of oxytocin in bovine systemic blood and luteal tissue between days 5 and 10 of the cycle. Maximal levels of luteal oxytocin detected on day 8 of the cycle probably represent, for the most part, stored oxytocin because greatest luteal mRNA levels for this nonapeptide are attained on day 3 and decline thereafter (Ivell et al., 1985). Results of the present study, however, differ from those of Schams et al. (1985a) who reported considerably lower oxytocin concentrations in bovine luteal tissue throughout the estrous cycle. In their study the corpus luteum was immediately frozen in liquid N_2 upon removal from the animal. In contrast, due to the nature of our study, some synthesis and(or) post-translational processing of hormone apparently occurred (see below) before the tissue was frozen.

Incubation of luteal tissue alone resulted in the release of

oxytocin in quantities that reflected the concentration initially present in the tissue. In vitro release of oxytocin from luteal tissue was greatest on day 8 of the cycle. After 2 h incubation total oxytocin concentration (tissue + medium) increased more than twofold. Even before incubation, the process of slicing and washing luteal tissue (30–40 min) increased oxytocin concentration by about 40%. This apparently does not represent active oxytocin synthesis because incorporation of (^{35}S)cysteine into bovine and ovine luteal oxytocin and putative oxytocin-precursor was demonstrated after 12 h of incubation only (Swann et al., 1984). As occurs in the hypothalamus, the synthesis of luteal oxytocin involves the formation of an approximately 14-K precursor protein, which is subsequently cleaved to form neurophysin and oxytocin (Swann et al., 1984). The observed increase in oxytocin concentration may therefore represent a post-translational processing of the prohormone.

Prostaglandin $F_{2\alpha}$ stimulated the in vitro release of oxytocin from luteal tissue on day 8 but not on days 12 and 16 of the cycle. In vitro release of oxytocin from day 8 luteal tissue increased in response to increasing levels of $PGF_{2\alpha}$ in the incubation medium. Failure of $PGF_{2\alpha}$ to stimulate additional secretion of oxytocin from day 12 luteal tissue in vitro is not entirely consistent with in vivo luteal response to exogenous hormone (Flint and Sheldrick, 1982; Walters et al., 1983). Similarly, the relatively large quantities of oxytocin secreted from the ovary around midcycle (Walters el al.,

1984; Schams et al., 1985b), presumably induced by an increase in uterine $PGF_{2\alpha}$ secretion (McCracken, 1984), might have contributed to the low levels of this "neuropeptide" present in luteal tissue on day 12 of the cycle in our study. If endogenous $PGF_{2\alpha}$ is responsible for reducing the luteal concentration of oxytocin, one would expect most luteal $PGF_{2\alpha}$ receptors to be saturated. Addition of low levels of $PGF_{2\alpha}$ (10ng/ml) to incubation media, as utilized in this study, would therefore be ineffective in inducing a significant release of oxytocin over control. Neither PGE_2 nor LH was effective in inducing the release of oxytocin from luteal tissue at any stage of the cycle studied. The mechanisms by which oxytocin is released from the ovary and the pituitary are apparently different. Contrary to its effect on release of neurohypophyseal oxytocin (Negro-Vilar et al., 1985), PGE_2 did not stimulate the release of ovarian oxytocin.

It has been postulated that oxytocin stimulates the release of $PGF_{2\alpha}$ from the uterus (Roberts et al., 1976) which in turn induces the secretion of ovarian oxytocin (Flint and Sheldrick, 1982) so that both hormones undergo a positive feedback action leading to complete luteolysis. However, this postulated scenario does not fit all the observations because results of this study and others (Schams, 1983; Wathes et al., 1984) indicate that oxytocin levels in the ovary and blood decline before luteal regression begins. In fact luteal regression occurs when oxytocin levels are lowest during the estrous cycle.

It is concluded that bovine luteal tissue actively synthesizes oxytocin that is available for immediate release in response to $PGF_{2\alpha}$. Based upon the results of the present study as well as data of others it appears that synthesis is most active during the first one-half of the cycle. Because luteal oxytocin is elevated early in the cycle it is possible that this hormone plays some role in development of the corpus luteum and(or) regulation of steroidogenesis. Oxytocin at low concentrations enhanced basal steroidogenesis in cultured luteal cells obtained from pregnant cows but at higher concentrations it inhibited progesterone production and the response of these luteal cells to hCG (Tan et al., 1982a,b). Similarly, oxytocin has been shown to inhibit LH-stimulated progesterone secretion from small ovine luteal cells leading to the postulate that it may serve as an intraluteal communicator between large and small cells (Niswender et al., 1985). A role for oxytocin in regulating luteal steroidogenesis is supported by recent findings that oxytocin is also synthesized in other steroidogenic tissues such as the testes and the adrenal cortex (Nicholson et al., 1984; Schams et al., 1985a).

EXPERIMENTS 3 AND 4: CYCLOHEXIMIDE, COLCHICINE AND CYTOCHALASIN B DO NOT AFFECT BOVINE LUTEAL OXYTOCIN SYNTHESIS AND RELEASE IN VITRO

INTRODUCTION

The neuropeptide hormone, oxytocin is synthesized by large luteal cells of the cow and ewe (Rodgers et al., 1983; Kruip et al., 1985; Fields and Fields, 1986) and stored in electron dense secretory granules (Rice and Thorburn, 1985; Guldenaar et al., 1985; Fields and Fields, 1986). In vitro exocytosis of these granules (Chegini and Rao, 1986) and secretion of oxytocin (Experiments 1 and 2) from bovine luteal slices is induced by $PGF_{2\alpha}$. It is generally accepted that cytoplasmic microtubules and microfilaments, elements of the cytoskeleton system, are involved in the process of peptide hormone secretion by interacting with the secretory granules to facilitate their transport to the plasma membrane and subsequently their release (Lacy et al., 1968; Williams and Wolf, 1970; Kraicer and Milligan, 1971; Olmsted and Borisy 1973; Lacy 1975).

A study of the kinetics of oxytocin synthesis and release by bovine luteal tissue showed that highest oxytocin concentrations were detected on day 8 of the estrous cycle and revealed a twofold increase in oxytocin levels after 2 h of incubation (Experiments 1 and 2). However, measurement of luteal oxytocin-specific mRNA throughout the estrous cycle of the cow showed that gene transcription was maximal accompanying ovulation and decreased

thereafter. Highest concentrations of mRNA were detected around day 3, declined sharply around day 7, reached basal levels by day 11 of the cycle and thereafter were only barely detectable (Ivell et al., 1985).

The present study was conducted to determine whether inhibition of de novo protein synthesis and the cellular cytoskeletal system would inhibit this twofold increase in oxytocin levels as well as the $PGF_{2\alpha}$ stimulatory effect on oxytocin release.

MATERIALS AND METHODS

Experiment 3

Effects of cycloheximide and $PGF_{2\alpha}$ on oxytocin synthesis and release in vitro from the day 8 bovine corpus luteum were investigated. Six Hereford and Hereford x Angus heifers (2 years old; 350-400 kg) were observed twice daily for estrus using vasectomized bulls. After exhibiting at least two consecutive estrous cycles of normal duration (18-23 days), heifers were slaughtered on day 8 of the estrous cycle (day of detected estrus = day 0 of the cycle).

Approximately 20 min after slaughter, the ovary bearing the corpus luteum was collected. The corpus luteum was placed into cold Eagle's deficient Minimum Essential Medium (MEM), containing 24 mM Hepes buffer, 100 U/ml penicillin, 100 ug/ml streptomycin, 2.5 ug/ml fungizone, 5 ug/ml insulin, 5 ug/ml transferrin and 5 ng/ml selenium. In addition the medium was supplemented with MEM non-essential amino

acids (Sigma). The corpus luteum was then transported to the laboratory (10 min), dissected from the ovarian stroma, weighed, sliced (0.3 mm thickness) and washed four times with 40 ml of the same medium. Slices were then blotted on a dampened filter paper and subsequently divided into 200-250 mg aliquots. Tissue aliquots were placed into one of five incubation flasks (prepared in duplicate) containing 2 ml of incubation medium (MEM, previously described, supplemented with 0.5 mM lysine, 0.1 mM methionine and 1.0 uCi/ml [U-^{14}C] L-leucine/ml (308 mCi/mmol, New England Nuclear, MA), to which was added the following treatments: 1,2) none, unincubated and incubated controls; 3) cycloheximide (0.355 mM); 4) prostaglandin $F_{2\alpha}$ (0.042 uM) and 5) cycloheximide + prostaglandin $F_{2\alpha}$. All flasks were flushed with 95% O_2-5% CO_2 and stoppered. Contents of unincubated control flasks were then transferred to glass tubes and centrifuged at 3000 x g, after which the tissue slices were separated from the supernatant and both were immediately frozen and stored at -20°C. Remaining flasks were incubated in a Dubnoff metabolic incubator at 38°C for 2 h. Incubation was terminated by immersing the flasks in ice water (4°C) after which they were processed as previously described for unincubated controls.

Experiment 4

Effects of cytochalasin B, colchicine and $PGF_{2\alpha}$ on *in vitro* oxytocin synthesis and(or) release from the bovine corpus luteum on day 8 of the estrous cycle was investigated. Five heifers similar to

those utilized in experiment 3 were slaughtered on day 8 of the estrous cycle. Corpora lutea were collected, sliced, washed and aliquots of 200-210 mg were placed into six pairs of flasks containing 2 ml of Ham's F-12 medium (Experiments 1 and 2). The following treatments were imposed: 1,2) none, unincubated and incubated controls, respectively; 3) colchicine (0.05 mM); 4) cytochalasin B (0.02 mM); 5) $PGF_{2}\alpha$ (0.042 uM) and 6) colchicine + $PGF_{2}\alpha$. Incubation of tissue was performed for 2 h as described in experiment 3.

Total Incorporation of [^{14}C]leucine into protein

Total incorporation of [^{14}C]leucine into protein was measured by a modification of the method described by Maurer and Gorski (1977). Tissue was homogenized in 2 ml of 1% acetic acid. The homogenate was vortexed, and a 1 ml aliquot was removed for determination of total incorporation of [^{14}C]leucine into protein. The remaining 1 ml was used for extraction of oxytocin from tissue. One milliliter of 10 mg/ml solution of bovine serum albumen was added to the former aliquot followed by 4 ml of distilled water and 5 ml of 10% trichloroacetic acid (TCA). The mixture was vortexed, left on ice for 5 min and then centrifuged at 800 x g for 10 min. The resulting pellet was washed twice by suspension in 2 ml of 0.2 M NaOH, addition of 2 ml of water and 5 ml of TCA followed by centrifugation. The final pellet was dissolved in 2 ml of tissue solubilizer (Amersham) for liquid scintillation counting.

Oxytocin Extraction and Radioimmunoassay

Oxytocin was extracted from the 1 ml of 1% acetic acid tissue homogenate as described above. The homogenate was centrifuged at 3000 x g for 10 min, the supernatant was freeze-dried and reconstituted in buffer as described (Experiments 1 and 2). Oxytocin in the incubation medium was assayed directly without extraction. Oxytocin in both tissue and incubation medium was measured by radioimmunoassay (Experiments 1 and 2). Intra-and interassay coefficients of variation were 3.6 and 9.6%, respectively.

Statistical Analyses

Statistical analyses were performed according to Snedecor and Cochran (1980). Data from experiments 3 and 4 were analyzed by two- and one-way analyses of variance, respectively.

RESULTS AND DISCUSSION

Cycloheximide inhibited ($P<0.01$) the incorporation of labeled leucine into newly synthesized protein by more than 90% (Table 3). This was consistent with the well documented effect of cycloheximide on protein synthesis (Wang and Greenwald 1985; Gokal et al., 1986; Matsui et al., 1986). However, oxytocin release into the medium and its retention in tissue slices after incubation was not affected by cycloheximide (Table 4). At the end of incubation, total oxytocin production (tissue + medium) was approximately twofold greater than that of unincubated control tissue levels. This increase in oxytocin

Table 3. Incorporation of [^{14}C]leucine (mean ± SE) in bovine luteal tissue *in vitro*

Treatment*	dpm/g tissue
Control	740560 ± 213470[a]
Cycloheximide	63200 ± 10890[b]
$PGF_{2\alpha}$	752290 ± 165530[a]
Cycloheximide + $PGF_{2\alpha}$	59830 ± 11510[b]

*Luteal tissue obtained from six heifers on day 8 of the estrous cycle was incubated with cycloheximide (0.355 mM), $PGF_{2\alpha}$ (0.042 um) and cycloheximide + $PGF_{2\alpha}$ for 2 h at 38°C.

[a,b]Means within a column with a different superscript letter differ ($P<0.05$).

concentration apparently does not represent de novo protein synthesis, because it could not be inhibited by cycloheximide. This is further substantiated by the fact that no ^{14}C radioactivity was extracted with oxytocin from tissue slices, which suggests that no incorporation of ^{14}C leucine into oxytocin occurred. These data are consistent with those of Swann et al. (1984) who reported that (^{35}S)cysteine was not incorporated in either ovine or bovine luteal oxytocin after 2 h of incubation. However, after 12 h (^{35}S)cysteine was recovered in both oxytocin and putative oxytocin precursor. Synthesis of luteal oxytocin involves the formation of an approximately 14-K precursor protein, which is subsequently cleaved to form neurophysin and oxytocin (Ivell and Richter, 1984; Swann et al., 1984; Ivell et al., 1985; Ivell, 1986). The observed increase in oxytocin concentration may therefore represent a post-translational processing of this prohormone because it occurred in the absence of incorporation of (^{14}C)leucine and while de novo protein synthesis was inhibited. This premise fits very well with the fact that luteal mRNA for oxytocin is very low on day 8 of the bovine estrous cycle (Ivell et al., 1985)

Prostaglandin $F_{2\alpha}$ provoked a significant release of luteal oxytocin over control, an effect which was not inhibited by cycloheximide or colchicine (Tables 3,4). This is in agreement with previous reports that $PGF_{2\alpha}$ stimulates oxytocin release in vitro from bovine corpora lutea on day 8 of the cycle (Experiments 1 and 2; Chegini et al., 1986). However, this $PGF_{2\alpha}$ effect was not consistent

Table 4. Oxytocin synthesis and(or) release by luteal tissue in response to cycloheximide and $PGF_{2\alpha}$ *in vitro*

Treatment*	Mean oxytocin concentration ($ng \cdot g^{-1} \cdot 2h^{-1}$)		
	Incubation medium	Tissue	Tissue + medium
Unincubated control	---	1806	---
Incubated control	1245[a]	1860	2936
Cycloheximide	1289[a]	1782	2894
$PGF_{2\alpha}$	1340[b]	1767	2956
Cycloheximide + $PGF_{2\alpha}$	1428[b]	1704	2996
Common SE	32	56	75

*Luteal tissue obtained from six heifers on day 8 of the estrous cycle was incubated with cycloheximide (0.355 mM), prostaglandin $F_{2\alpha}$ (0.042 um) and cycloheximide + prostaglandin $F_{2\alpha}$ for 2 h at 38°C.

[a,b]Means within a column with a different superscript letter differ ($P<0.05$).

Table 5. Oxytocin synthesis and(or) release by luteal tissue in response to colchicine, cytochalasin B and $PGF_{2\alpha}$ *in vitro*

Treatment*	Mean oxytocin concentration ($ng \cdot g^{-1} \cdot 2h^{-1}$)		
	Incubation medium	Tissue	Tissue + medium
Unincubated control	—	1141	—
Incubated Control	1089[a]	1040	2095
Colchicine	1093[a]	1106	2163
Cytochalasin B	1087[a]	1060	2274
$PGF_{2\alpha}$	1234[b]	1045	2201
Colchicine + $PGF_{2\alpha}$	1178[b]	1119	2309
Common SE	55	142	199

*Luteal tissue obtained from five heifers on day 8 of the estrous cycle was incubated with colchicine (0.05 mM), cytochalasin B (0.02 mM), $PGF_{2\alpha}$ (0.042 uM) and 6) colchicine + $PGF_{2\alpha}$ for 2 h at 38°C.

[a,b]Means within a column with a different superscript letter differ ($P<0.05$).

with the results of Hirst et al. (1986) who found that $PGF_{2\alpha}$ had no effect on oxytocin release from ovine luteal tissue in vitro. Prostaglandin $F_{2\alpha}$ also failed to induce oxytocin release from bovine luteal slices on days 12 and 16 of the estrous cycle (Experiment 1). Prostaglandin $F_{2\alpha}$ may therefore not be universally effective in stimulating the release of oxytocin from luteal tissue.

Neither cholchicine nor cytochalasin B inhibited oxytocin release into the medium or its retention in tissue slices after incubation (Table 5). Colchicine reportedly disrupts microtubules through direct binding to tubulin dimers (Borisy and Tayler, 1967; Wilson et al., 1974; Margolis and Wilson, 1977) and blocks the release of histamine (Gillespie et al., 1968), thyroxine (Williams and Wolff, 1970), adrenocorticotropic hormone (Kraicer and Milligan, 1971) and insulin (Lacy et al., 1968). Failure of colchicine to block oxytocin release in the present study may be due to the short duration of incubation. At low concentrations, colchicine equilibrium with tubulin is not reached until 6-8 h (Wilson et al., 1974). Preincubation or incubation for a longer time may therefore be necessary to elicit a colchicine effect on oxytocin release. Microfilaments, actin-containing components of cytoskeleton, are thought to play an important role in the secretion of hormones. Cytochalasin B, a drug that inhibits microfilament polymerization, blocked gonadotropin-releasing hormone (GnRH)-induced LH release (Khar et al., 1979; Adams and Nett, 1979; Pickering and Fink, 1979; Lewis et al., 1985). In contrast, Liu and Jackson, (1986)

demonstrated that cytochalasin B did not effect either basal or GnRH-stimulated LH release but inhibited GnRH-stimulated LH glycosylation. In the present study cytochalasin B had no effect on either basal or $PGF_{2\alpha}$-stimulated oxytocin release.

It is concluded that short term release of luteal oxytocin *in vitro* is neither contingent upon *de novo* protein synthesis nor can it be interrupted by exposure to drugs that inhibit cytoskeletal integrity. Over a period of 2 h, oxytocin synthesis and release by bovine luteal tissue on day 8 of the estrous cycle may depend mainly on the post-translational processing of oxytocin prohormone.

GENERAL DISCUSSION

Factors that regulate oxytocin synthesis and release from the bovine and ovine corpus luteum have recently received considerable attention. In this section an attempt will be made to discuss the results of the four experiments conducted in the present research and compare them with results of research from other laboratories. Results of Experiment 1 of the present study demonstrated that luteal oxytocin concentrations in the cow were highest on day 8 of the estrous cycle. These data are consistent with the results of Wathes et al. (1984) and Sheldrick and Flint (1983) who reported that the greatest concentrations of luteal oxytocin were found during the early luteal phase of the cycle in the cow and ewe, respectively.

In Experiment 1, $PGF_{2\alpha}$-induced a significant *in vitro* release of oxytocin from bovine luteal tissue collected on day 8 of the estrous cycle. Similarly, Chegini and Rao (1986) found that $PGF_{2\alpha}$-induced migration and exocytosis of electron-dense secretory granules that contained oxytocin (Rice and Thorburn, 1985; Fields and Fields, 1986) from bovine large luteal cells *in vitro*. The results of the present study also agree with those of Barrett et al. (1987) who found low doses of $PGF_{2\alpha}$ to cause a slight increase in oxytocin release from bovine luteal cells cultured for 24 h. However, these results are not consistent with those of Hirst et al. (1986) who reported that $PGF_{2\alpha}$ had no effect on oxytocin release from ovine luteal slices on days 8-10 of the estrous cycle. In addition, $PGF_{2\alpha}$ failed to induce

a significant release of oxytocin *in vitro* from bovine corpora lutea on days 12 and 16 of the cycle in Experiment 1. The effect of prostaglandin $F_{2\alpha}$ on oxytocin release from luteal cells may therefore vary with the stage of the estrous cycle.

Luteinizing hormone and PGE_2 had no effect on oxytocin release at any stage of the cycle studied. These results agree with those of Chegini and Rao (1986) and Hirst et al. (1986) who could find no effect of these hormones on oxytocin release from bovine and ovine luteal tissue *in vitro*. However, they are not consistent with the results of Barrett et al. (1987) who reported that LH was capable of stimulating oxytocin release from bovine luteal cells in culture. This suggests that a longer time (24 h) may be needed to elicit LH-induced oxytocin release *in vitro*. In Experiment 1 and in the studies of Chegini and Rao (1986) and Hirst et al. (1986) luteal slices were incubated for 2 h only, which may not have been of sufficient duration to demonstrate the effect of LH.

Oxytocin synthesis by bovine luteal slices was clearly demonstrated in Experiments 1,2,3 and 4. These data have been confirmed by Barrett et al. (1987). However, increases in oxytocin concentrations by bovine luteal slices on day 8 of the cycle does not appear to represent *de novo* synthesis because it occurred in the absence of incorporation of labeled leucine and during the inhibition of *de novo* protein synthesis. Ivell et al. (1985) found that oxytocin mRNA concentrations in bovine luteal tissue were highest on day 3 of the cycle, very low on day 7 and reached basal levels by day

11. This suggests that the major portion of oxytocin in the bovine corpus luteum is synthesized around day 3 of the cycle and stored in the form of oxytocin prohormone which is processed further during later stages.

Further research should be undertaken to elucidate the factors that regulate the expression of oxytocin gene as well as oxytocin synthesis and release from the corpus luteum. Furthermore, the effects of oxytocin on the corpus luteum function and the estrous cycle in general need to be examined.

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