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A series of experiments were conducted to characterize the isozymes of protein kinase C (PKC) present in the bovine corpus luteum (CL) and their subcellular distribution. In addition, the functional interrelationships between PKC, calpains and calpastatin during *in vivo* prostaglandin $F_{2\alpha}$-induced secretion of oxytocin (OT) from the bovine CL were evaluated. Finally, the ability of the myrisotylated alanine rich C kinase substrate (MARCKS) to serve as a substrate for PKC during the $PGF_{2\alpha}$-induced secretion of OT was determined. **Exp. 1:** Research was conducted to determine the nature of the isoforms of protein kinase C (PKC) present in the bovine (*Bos taurus*) corpus luteum (CL) and their subcellular distribution. Western blot analysis was performed using isozyme-specific polyclonal antibodies, which revealed that the bovine corpus luteum contains the α and ε isozymes of PKC, but not the β, γ, δ or ζ isozymes. Subcellular fractionation demonstrated that the α and ε isozymes of PKC were present in both the cytosolic and plasma membrane fractions. Densitometric measurements of Western blots of the

subcellular luteal PKC fractions indicated that PKCα was found predominantly in the cytosolic fraction, whereas, the majority of PKCε was associated with the plasma membrane. **Exp. 2:** An experiment was conducted to determine whether prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced secretion of oxytocin (OT) by the bovine corpus luteum (CL) was associated with changes in the activities of protein kinase C (PKC), calpains and calpastatin. On day 8 of the estrous cycle (estrus = day 0), beef heifers were restrained and given a 500 μg i.v. injection of cloprostenol, a $PGF_{2\alpha}$ analog. Corpora lutea were surgically removed from beef heifers at 0, 2, 7.5 or 30 min (n = 4 animals per time period) after cloprostenol injection. Blood samples were collected before injection and at frequent intervals after injection. Relative to mean plasma levels of OT at time 0, peak plasma levels occurred between 1.5 and 10 min for all animals. Mean luteal concentration of OT was greater at 0, 2 and 7.5 min than at 30 min, but differences in tissue OT over time were not significant ($p>0.05$). Protein kinase C activities in the membrane or cytosolic fractions did not differ significantly among the times of CL removal; however, membrane PKC activity was positively correlated with plasma level of OT at time of CL removal ($r = 0.82$, $p<0.0025$). Luteal m-calpain and μ-calpain activities did not change over time after $PGF_{2\alpha}$ ($p>0.05$) and calpastatin activity was significantly higher at 30 min than at 0, 2, or 7.5 min ($p<0.05$). **Exp. 3:** Western blot analysis revealed that MARCKS is present in the day 8 bovine corpus luteum. Both 12-O-tetradecanoylphorbol-13-acetate (TPA) and $PGF_{2\alpha}$ caused an increase in the phosphorylation of MARCKS in membrane and cytosolic fractions from luteal tissue slices. A model is proposed describing the involvement of MARCKS in the $PGF_{2\alpha}$-induced secretion of OT from the bovine corpus luteum.

PROSTAGLANDIN $F_{2\alpha}$-INDUCED SIGNAL TRANSDUCTION MECHANISM REGULATING THE SECRETION OF OXYTOCIN FROM THE BOVINE CORPUS LUTEUM

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LITERATURE REVIEW

Of all mammalian embryos, 25-55% are lost early in gestation and at least some of this loss appears to be due to inadequate function of the corpus luteum (Niswender and Nett, 1988). Efforts to understand the regulation of the life span and function of this transient endocrine gland have important implications for improving reproductive efficiency in domestic animals. The research in this thesis represents an attempt to examine the functional luteal intracellular biochemical events involved in regulating the secretion of oxytocin from the bovine corpus luteum.

Hormonal Control of the Estrous Cycle and Early Pregnancy

The corpus luteum is a transient endocrine gland in the ovary that is derived from the cells of the ovulated follicle. The primary function of this gland is to secrete progesterone, which acts on several target tissues in the reproductive system to prepare them to support a pregnancy should the ovulated ovum be fertilized. If the ovum is not fertilized, the mammalian uterus secretes prostaglandin $F_{2\alpha}$, which brings about the regression of the corpus luteum resulting in enhanced sexual behavior and the ovulation that heralds the onset of the ensuing estrous cycle.

Regulation of the function of this ephemeral endocrine gland is necessarily complex and involves regulation by hormones emanating from several other tissues, including the hypothalamus, pituitary, uterus and ovary. These hormones may act directly or indirectly on the corpus luteum to stimulate (luteotropic) or inhibit (luteolytic)

progesterone synthesis and secretion, which is universally used as a measure of luteal function.

The estrous cycle in farm animals is composed of a follicular and a luteal phase and these two broad classifications can be further divided into four stages: proestrus, estrus, metestrus and diestrus. Each stage is defined by the fluctuating levels of the hormones of the estrous cycle. These hormones include gonadotropin releasing hormone (GnRH) from the hypothalamus, luteinizing hormone (LH) and follicle stimulating hormone (FSH) of hypophyseal origin, estrogen and progesterone from the ovary and prostaglandins secreted by the uterus. In farm animals, the beginning of the estrous cycle is defined as the time of onset of behavioral estrus, so it is deemed appropriate to begin a discussion of the estrous cycle by describing the events during proestrus that promote the onset of estrus and the subsequent ovulation.

Folliculogenesis

Ovarian follicles develop in waves and in cows there are typically three waves of follicular growth during one estrous cycle. Each wave is characterized by the development of one large dominant follicle and several non-dominant follicles (Sirois and Fortune, 1984). The hormonal profile for the follicles that develop during the first and second wave is not appropriate and these follicles undergo atresia (Sorois and Fortune, 1988). However, the third wave of follicles develops in a hormonal environment (proestrus) that stimulates the maturation of one or more tertiary follicles into Graffian follicles capable of ovulating an oocyte. Systemic levels of FSH and LH increase concomitantly at or near the onset of estrus in cows (Akbar et al., 1974) and ewes (Pant et al., 1977). Approximately 24 hours after the initial increase in gonadotropins (LH and FSH), 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 in FSH may play a role in follicle maturation. In this regard, Ireland (1987) demonstrated that short-term hypophysectomy inhibits the development of follicles and administration of FSH restores follicular development in the rat. Follicle stimulating hormone causes granulosa cell mitosis and follicular fluid formation, an activity that is enhanced by the secretion of estradiol-17β produced by the granulosa cells of the follicle. In addition, FSH increases granulosa cell sensitivity to LH by causing the up-regulation of LH receptors (Hsueh, 1984).

The mechanism by which one follicle dominates and suppresses the growth of other non-dominant follicles is not well understood, but may involve intragonadal regulation by components of follicular fluid in the dominant follicle. Inhibin was first discovered in bovine follicular fluid by DeJong and Sharp (1976) and is a strong negative regulator of FSH secretion (Ying, 1988; Hillier, 1991). *In vivo* administration of bovine follicular fluid (containing inhibin only) suppressed the production of messenger RNA for the β-subunit of FSH in the pituitary and decreased circulating concentrations of this gonadotropin (Beard et al., 1989). It has been suggested that inhibin produced by the dominant follicle may inhibit the growth of sub-dominant follicles by depriving them of the FSH required to promote their development (Richards, 1980).

The preovulatory Graffian follicle produces high levels of estrogen which has two effects in the brain. In farm animals the increased levels of estrogen act on the higher centers of the brain to stimulate estrous behavior (Herbert, 1972). Unlike humans, livestock species are only receptive to mating during estrus. Duration of estrus varies from species to species and for ewes lasts 24-60 hours; cows, 18-19 hours; does, 32-40 hours; sows, 48-72 hours and mares, 4-8 days (Levasseur and Thibault, 1980). In addition to stimulating estrous behavior estrogen evokes the release of GnRH from the hypothalamus, which causes a surge of luteinizing hormone to be released from the anterior pituitary. In this regard, It has been demonstrated that administration of exogenous estradiol elicited a preovulatory-like surge of LH in ewes (Howland et al.,

1971) and cows (Beck and Convey, 1977). Estradiol caused GnRH release from the hypothalamus (Kesner et al., 1981) and increased the sensitivity of the pituitary to GnRH, resulting in an increased quantity of LH released in response to consecutive standard doses of GnRH *in vitro* (Reeves et al., 1971; Kesner et al., 1981). Each pulse of LH is followed by an increase in estrogen in the ovarian venous blood (Baird et al., 1976) which presumably acts by a positive feedback mechanism to stimulate further release of LH. Both increased release of GnRH from the hypothalamus and increased sensitivity of the anterior pituitary to GnRH are required to elicit the preovulatory surge of LH (Kesner and Convey, 1982). This LH surge is characterized by pulses of increasing magnitude and frequency in the ewe (Baird, 1978) and cow (Rahe et al., 1980), resulting in sufficient systemic concentrations of the gonadotropin to cause ovulation.

It has been suggested that the positive feedback effect of estrogen only occurs as progesterone concentrations from the previous cycle decline. Estrogen did not cause an LH surge in animals that had fully functional corpora lutea (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). These results are supported in part by the data of Howland et al. (1971) who demonstrated that an i.m. injection of 750 μg estradiol-17β into ewes at mid-cycle failed to induce gonadotropin release. However, these researchers found that injection of 2 mg of estradiol caused a small release of LH in mid-cycle ewes, suggesting that a higher pharmacological dose of estrogen may be able to overcome the inhibitory effect of progesterone.

Mechanism of ovulation

A cascade of biochemical changes occur as a result of the preovulatory LH surge. The preovulatory gonadotropin surge causes a local increase in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2), and inhibition of prostaglandin synthesis has been

shown to prevent ovulation (Armstrong, 1975). The effect of these eicosanoids is exerted at the level of the follicular wall epithelium where they stimulate the activity of collagenase and other proteolytic enzymes that cause the rupture of the follicle and the release of the oocyte (Rondell, 1970; Espey, 1978). Inhibition of prostaglandin synthesis with the cyclooxygenase inhibitor indomethacin has been shown to prevent ovulation in rodents (Eckholm et al., 1982) and sheep (Murdoch et al., 1986; Murdoch and McCormick, 1991). Murdoch et al. (1986) reported that the inhibitory effect of indomethacin could be reversed by systemic injection of exogenous PGE_2 or $PGF_{2\alpha}$.

The preovulatory LH surge also stimulates steroid production by the preovulatory follicle (Lipner, 1988). Treatment of perfused rat ovaries with the aromatase inhibitor, 4-hydroxyandrostenedione, inhibited LH-induced estrogen production, but did not prevent ovulation (LeMaire, 1989). Similar studies using the 3β-hydroxysteroid dehydrogenase inhibitor 17α-(3'-hydroxypropyl)-1,3,5,6,8(9)-oestropentaene-3,17β-diol on perfused rat ovaries inhibited ovulation. This effect could be reversed by treatment with progesterone but not testosterone (Brännström and Janson, 1989). In addition, immunization of pregnant mare serum gonadotropin (PMSG)-primed immature rats against progesterone results in the inhibition of ovulation (Mori et al., 1977). Collectively, these data support a role for ovarian progesterone, but not estrogen or testosterone in ovulation.

Termination of the preovulatory gonadotropin surge results from refractoriness of the pituitary to GnRH (Chakraborty et al., 1974; Kesner and Convey, 1982) and not a depletion of gonadotropin content (Convey et al., 1981). Time of ovulation varies among species and for ewes occurs 24-30 hours from the beginning of estrus; does, 30-36 hours from the beginning of estrus; sows, 35-45 hours from the beginning of estrus; cows, 10-11 hours after the end of estrus; and mares, 1-2 days before the end of estrus (Levasseur and Thibault, 1980).

Formation of the corpus luteum

After ovulation, LH causes the differentiation or luteinization of the cells of the ovulated follicle which results in the formation of the corpus luteum. Metestrus is the period immediately after estrus when estrogen levels from the Graffian follicle are declining and progesterone levels from the developing corpus luteum are increasing. Diestrus is characterized by the presence of a functional corpus luteum that produces progesterone. The corpus luteum achieves it's full size by 7-9 days after ovulation in ewes (Duncan et al., 1960), 12 days after ovulation in the cow and 6-8 days after ovulation in the sow (Erb et al., 1971). The size of the ruminant corpus luteum is correlated with its ability to produce progesterone (Hafs and Armstrong, 1968; Hansel et al., 1973). In contrast, the mare corpus luteum reaches its full size within 3 days after ovulation, but maximal secretion of progesterone does not occur until 9 days after ovulation (Nett et al., 1976b).

Effect of progesterone on the female reproductive system

Progesterone has several effects on target tissues in the reproductive tract to prepare them to support a pregnancy if the ovulated ovum is fertilized. In order for progesterone to have an effect on the female reproductive tract, the cells must first have been exposed to estrogen which induces the formation of receptors for progesterone (Muldoon, 1980). In the estrogen-primed system, progesterone causes contractions in the oviduct that influence the rate of transport of the ovum to the uterus (Blandau et al., 1977). Progesterone also causes the secretion of fluid in the oviduct that supports the early development of the zygote. Luteal progesterone has several significant effects at the level of the uterus. The uterine mucosa thickens due to significantly increased vascularity of the stroma (Porter and Finn, 1977). Under the influence of estrogen, the cervix

secretes a mucus rich in glycoprotein that facilitates the passage of sperm into the uterus. Progesterone causes the consistency of the cervical mucus to become highly viscous and inhibit the movement of substances into and out of the uterus. Therefore, under the influence of progesterone the cervix provides an effective barrier between the uterus and the external environment, thus serving to protect the developing cenceptus (Porter and Finn, 1977).

Progesterone also has a direct effect on the GnRH pulse generator in the hypothalamus. This effect is manifested by pulses of LH from the anterior pituitary that are less frequent, but of higher amplitude than during the follicular phase of the cycle (Baird and Scaramuzzi, 1976; Karsch et al., 1979; Rahe et al., 1980). Luteinizing hormone released during the luteal phase of the cycle in ruminants is luteotropic and stimulates the release of progesterone from the corpus luteum. This premise is supported by Kaltenbach et al. (1968) who demonstrated that hypophysectomy of ewes on day 1 after ovulation resulted in failure of the corpus luteum to form and hypophysectomy on day 5 caused premature luteal regression. However, infusion of a crude pituitary preparation containing LH and FSH maintained luteal function in both pregnant and non-pregnant ewes. Subsequent studies by Karsch et al. (1971) showed that constant infusion of LH prolonged the life span and function of the corpus luteum in intact, cycling ewes. Injections of LH lengthened the estrous cycle in the cow (Donaldson and Hansel, 1965), prevented oxytocin-induced early luteolysis (Simmons and Hansel, 1964; Donaldson et al., 1965), and increased luteal progesterone concentrations *in vivo* (Brunner et al., 1969) and *in vitro* (Hansel, 1971). Furthermore, daily injections of antiserum against LH reduced luteal weight and content of progesterone in intact and hysterectomized heifers (Snook et al., 1969).

Non-fertile cycle

If the ovulated ovum is not fertilized, the uterus secretes prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which acts back on the ovary to cause regression of the corpus luteum by mechanisms that will be discussed in detail (McCracken et al., 1972). Regression of the corpus luteum results in a decrease in systemic progesterone, which allows for follicular development, the preovulatory LH surge and the ovulation of the oocyte for the ensuing estrous cycle.

This has been a general review of the hormonal regulation of the estrous cycle in ruminants. However, it should be recognized that different species have different estrous cycle characteristics.

In rats, the secretion of progesterone during the estrous cycle is short-lived and at least on the first day of diestrus is controlled from within the corpus luteum. By the second day of diestrus pituitary gonadotropin is required to stimulate the release of the steroid, but unlike ruminants, the primary pituitary luteotropin in rats is prolactin, not LH (Rothchild, 1965).

Rabbits do not exhibit true estrous cycles. In this species physical stimulation of the cervix is required to induce ovulation and the preovulatory surge of LH occurs as a result of this stimulation (Keyes et al., 1983). If the ovulatory stimulus is not a fertile mating, a pseudopregnancy results and corpora lutea secrete progesterone for 16-17 days (Hillard et al., 1974). Follicular estrogen secretion, stimulated by luteinizing hormone, is required for maintenance of the corpus luteum in this species. Chronic treatment of intact pseudopregnant rabbits with estradiol-17β stimulates luteal secretion of progesterone, but has no effect on the life span of the corpus luteum (Miller and Keyes, 1976; Bill and Keyes, 1983).

Removal of the pituitary gland from pigs during the first two days of the estrous cycle is not detrimental to the corpus luteum. Therefore, it appears that the porcine corpus luteum can function without gonadotropin support after the initial LH surge at ovulation (Duncan et al., 1961).

Corpora lutea of most species are composed of large and small cells that are derived from the theca and granulosa cells of the ovulatory follicle. In contrast, mare corpora lutea develop exclusively from the granulosa cells (Harrison, 1946; Van Niekerk et al., 1975). Similar to luteal development in ruminants, the equine corpus luteum is dependent primarily on LH, because treatment with antisera against the gonadotropin results in premature luteal regression (Pineda et al., 1973).

Humans and non-human primates exhibit menstrual cycles instead of estrous cycles. Day 0 of the primate cycle is defined as the first day of menses and ovulation occurs at mid-cycle, about day 14 in the human. Continued low levels of LH are required for the maintenance of luteal function and for progesterone synthesis and release (Vande Wiele et al., 1970).

Early gestation

Mammalian species exhibit a wide variety of requirements for the presence of a corpus luteum during pregnancy. In order for the corpus luteum to escape luteolysis and remain viable during pregnancy, some luteotropic or antiluteolytic signal is required. The corpora lutea of pregnant rabbits, sows and does exhibit an absolute requirement for luteotropic stimuli from the anterior pituitary (reviewed by Stormshak et al., 1987). Hypophysectomy in these species always results in luteolysis.

Rats, mice and hamsters require pituitary luteotropins for the first half of gestation, after which placental proteins maintain the corpus luteum. The rat decidua also produces a prolactin-like protein that plays a luteotropic role early in gestation (Jayatilak et al.,

1985; Herz et al., 1986). Decidual prolactin expression declines at about mid-gestation, when the placenta begins to produce the luteotropic stimulus (Kelly et al., 1975).

The placenta acquires the ability to synthesize progesterone during gestation in the ewe, cow and mare, precluding the need for the corpus luteum (reviewed by Heap and Flint, 1984). In addition, ovine and bovine conceptuses produce trophoblastic proteins that suppress estradiol-stimulated $PGF_{2\alpha}$ secretion from the uterus that normally occurs during luteolysis (Godkin et al., 1982; Thatcher et al., 1985).

The sow has an absolute requirement for the corpus luteum throughout pregnancy and progesterone replacement therapy is sufficient to maintain pregnancy in ovariectomized sows (Ellicott and Dzuik, 1973). In this species, the blastocysts begin to synthesize estrogen at about days 9-12 of gestation (Pope et al., 1982; Bazer et al., 1986). The luteotropic effect of estrogen in this species acts indirectly by causing endometrial $PGF_{2\alpha}$, which would otherwise cause luteal regression, to be sequestered in the uterine lumen (Bazer and Thatcher, 1977; Bazer et al., 1986).

In pregnant humans, nonhuman primates and guinea pigs a corpus luteum is required throughout pregnancy, but hypophysectomy has no detrimental effect because the placenta produces the luteotropic stimulus (Stormshak et al., 1987). The luteotropin in women and nonhuman primates is chorionic gonadotropin that is produced very early in gestation by the preimplantation blastocysts (Hearn, 1986). Human chorionic gonadotropin (hCG) binds LH receptors in the corpus luteum (CL) and replaces LH as the luteotropic hormone that rescues this gland from $PGF_{2\alpha}$-induced regression (Hearn, 1986).

General Characteristics of the Corpus Luteum

The morphological characteristics of the corpus luteum have been studied for a number of species including the cow, ewe, sow, rabbit and primate. The corpus luteum

consists of two major steroidogenic cell types, designated small cells (10-22 μm) and large cells (22 to greater than 35 μm) based on size. This is not surprising considering the fact that the CL is derived from at least two steroidogenic cell types (granulosa and theca interna) of the ovulatory follicle (Corner, 1919; Warbritton, 1934; Mossman and Duke, 1973; Niswender et al., 1985). On a volume basis large cells make up about 25-35% of the ovine corpus luteum and small cells constitute approximately 12-18% of luteal volume during the period of maximum secretion of progesterone. The corpus luteum also contains vascular elements that account for approximately 11% of luteal volume. The remainder of the corpus luteum is composed of connective tissue (22-29%) and fibroblasts (7-11%, Nett et al., 1976a; Rodgers et al., 1984). A similar study of corpus luteum cell composition on a volume basis has not been performed in the cow. However, on a cell number basis, Lei et al. (1991) demonstrated that the mid-luteal phase bovine corpus luteum is composed of 22% small cells, 18% large cells and 60% non-luteal cells.

Follicular origins of large and small luteal cells

In a review of histological investigations of the corpus luteum, Corner (1915) suggested three hypotheses for the origin of cells of the corpus luteum. The first premise was that both luteal cell types were derived from the theca interna cells of the follicle. Conversely, it was suggested that both luteal cell types were derived from the granulosa cells of the follicle. A third possibility was that cells of the corpus luteum are derived from both follicular cell types. In 1919, Corner described ovulation and corpus luteum formation in the sow and Rhesus monkey and concluded that both granulosa and theca interna cells of the follicle gave rise to the cells of the corpus luteum. This research was advanced one step further in studies describing corpus luteum formation (McNutt, 1924; Gier and Marion, 1961) and the corpus luteum of pregnancy in the cow (McNutt, 1927; Foley and Greenstein, 1958). These investigators concluded that the small cells of the

corpus luteum were produced from the theca interna cells and the large cells were derived from the granulosa cells.

Results of a histological study by Donaldson and Hansel (1965) support the theory that the small and large cells of the corpus luteum are derived from both steroidogenic cells types of the follicle, but did not support the contention that small cells are theca-derived and large cells are granulosa-derived. Based on analysis of mitotic activity in the two cell types, these researchers suggested that granulosa-derived cells are of limited potential, developing into functional luteal cells by day 4 of the estrous cycle. Further growth of the CL occurs as a result of division and enlargement of theca-derived cells, which are responsive to exogenous gonadotropin after day 4 of the cycle and develop into medium and large luteal cells.

More recently, Alila and Hansel (1984) developed specific monoclonal antibodies to granulosa and thecal cell surface antigens to determine the contributions of these two follicular cell types to the cells of the corpus luteum. In the developing CL they found that the granulosa antibody bound mainly to large cells and the theca antibody bound to small cells. However, as the CL aged, the number of large cells stained with the granulosa antibody declined and by mid-cycle one-half of the large cells were labeled with the thecal antibody. By day 100 of gestation, no granulosa-derived large cells remained in the CL. This study confirmed previous reports that theca-derived small luteal cells can develop into large cells in the cow (Donaldson and Hansel, 1965) and the ewe (Warbritton, 1934; Fitz et al., 1982) and that granulosa-derived large cells have a limited life span.

Biochemical comparison of large and small luteal cells

Appreciation of the biosynthetic potential of these two cell types has only recently been discovered as advances in technology have allowed the isolation of relatively pure populations of small and large luteal cells from cows (Ursely and Leymarie, 1979; Koos

and Hansel, 1981), sheep (Rodgers and O'Shea, 1982; Niswender et al., 1985), pigs (Lemon and Loir, 1977), primates (Hild-Patito et al., 1986) and rabbits (Hoyer et al., 1986).

Both luteal cell types share several structural features that are typical of steroid secreting cells, including numerous mitochondria, abundance of smooth endoplasmic reticulum and large lipid droplets (Christensen and Gillim, 1969). Indeed, it is well documented that the large and small cells of the corpus luteum synthesize and secrete progesterone (Lemon and Loir, 1977; Ursely and Leymarie, 1979; Koos and Hansel, 1981; Fitz et al., 1982; Rodgers and O'Shea, 1982). The small cells are spindle shaped, have dark staining cytoplasm and an irregularly shaped nucleus (O'Shea et al., 1979). In contrast, large cells are polyhedral in shape with lightly staining cytoplasm and a large centrally located nucleus with a distinct nucleolus (Deane et al., 1966; Enders, 1973). In addition to the morphological differences between these two steroidogenic cell types, there are several biochemical differences.

Although both luteal cell types produce progesterone, the mechanism stimulating the release of this steroid is apparently different. Using an elutriation system to separate large and small luteal cell fractions, Fitz et al. (1982) reported that ovine large luteal cells produced approximately 20 times more progesterone than small cells when maintained in the unstimulated state. However, upon addition of LH or cAMP, the rate of progesterone secretion was significantly increased in small cells while large cells were only minimally affected. This observation is in agreement with the results of Rodgers and O'Shea (1982) who used a ficoll gradient to separate the two luteal cell types. However, these results do not agree with similar studies performed with CL from the pig (Lemon and Loir, 1977) and cow (Ursely and Leymarie, 1979; Koos and Hansel, 1981) in which large cells did respond to LH with an increase in progesterone production. But, in these latter studies, purity of the large cell fractions were either poor or not reported and approximately 1000

times as much LH was required to elicit maximal progesterone production from large cells as compared with small cells.

Fitz et al. (1982) also demonstrated that small cells had approximately 10 times more LH/hCG binding sites per cell than large cells in corpora lutea of superovulated sheep. In contrast, on a per cell basis, large cells bound 30 times more $PGF_{2\alpha}$ and 10 times more PGE_2 than small cells. In addition, receptors for estradiol in the ewe are five times more abundant in large cells than in small cells (Glass et al., 1984). These data indicate that the luteotropic effects of LH on the CL must be mediated through the small cells and the luteotropic (PGE_2) and luteolytic ($PGF_{2\alpha}$) effects of the prostanoids must be mediated by interactions with receptors on the large cells; thus, implying that there are paracrine interactions between the large and small cells of the corpus luteum. The balance between these luteolytic and luteotropic effects is essential for regulation of luteal function (Khan-Dawood and Dawood, 1986).

The difference in the number of receptors for these four biochemicals in the large and small luteal cells have important implications for the regulation of luteal function throughout the cycle. The corpus luteum undergoes dynamic changes in the proportions of large and small luteal cells during the estrous cycle (Thwaites and Edey, 1970; Fitz et al., 1981; O'Shea et al., 1986; Farin et al., 1986). Large cells are present only in small numbers in the early corpus luteum (before day 5 of the cycle) and their numbers increase until mid-cycle when large cells make up a greater volume of the CL than small cells. Consequently the relative responsiveness of the corpus luteum to LH, $PGF_{2\alpha}$, PGE_2 and estradiol changes as luteal development advances. It should be noted, however, that we classify cells small or large based purely on size determination. It is conceivable that some small cells of the early corpus luteum have adopted some of the biochemical characteristics of large cells, but have not yet achieved large cell size.

In addition to their steroid secreting potential, large cells also possess the cellular machinery found in cells specialized for the secretion of polypeptide and protein

hormones, including numerous Golgi complexes, rough endoplasmic reticulum and secretory granules (Niswender et al., 1985). Secretory granules measuring 0.2-0.4 μm in diameter are abundant throughout the cytoplasm of large cells, but are virtually absent in small cells. Although secretory granules are similar to lysosomes and peroxisomes in size and appearance, they are recognized as a distinct class of cytoplasmic granule because they are released at the cell surface by exocytosis and are devoid of acid phosphatase or catalase activities (Gemmell et al., 1974). Exocytosis of secretory granules from the large cells of the corpus luteum is correlated with the secretion of progesterone both *in vivo* (Gemmell et al., 1974) and *in vitro* (Sawyer et al., 1979). Several investigators have suggested that these granules contain progesterone and a progesterone binding protein and represent the mode of progesterone release from these cells (Gemmell et al., 1974; Sawyer et al., 1979; Quirk et al., 1979; Parry et al., 1980). However, if this is truly the mechanism by which progesterone is secreted from large cells, it leaves no explanation for how this steroid is released from small luteal cells. Other investigators argue that once progesterone is synthesized it leaves the cell by diffusion (Enders, 1973; Carlson et al., 1983; Rice et al., 1986). It is more facile to reconcile this means of progesterone release with the two different cell types.

The ovaries of domestic animals and primates produce several protein or peptide hormones including relaxin (Anderson and Long, 1978; Kendall et al., 1978; Fields et al., 1980; Fields and Fields, 1985), GnRH-like ovarian hormone (Aten et al., 1986), oxytocin (Rodgers et al., 1983; Wathes et al., 1983; Guldenaar et al., 1984; Ivell and Richter, 1984a; Khan-Dawood, 1986), neurophysin (Wathes et al., 1983; Fields and Fields, 1986; Khan-Dawood, 1986) and possibly vasopressin (Wathes et al., 1983). It is assumed that these neuropeptides are all contained within the secretory granules of the large luteal cells, though this has only been conclusively demonstrated for relaxin (Kendall et al., 1978; Fields and Fields, 1985), oxytocin (Rice and Thorburn, 1985; Fields et al., 1992) and

neurophysin (Field and Fields, 1986). Mechanisms of synthesis and secretion of OT from the corpus luteum will be discussed.

Oxytocin

Historical perspective

It has long been recognized that products of the posterior pituitary have the oxytocic qualities that stimulate smooth muscle contraction (Dale, 1906) and milk ejection (Ott and Scott, 1910). The value of these properties were almost immediately recognized and put to clinical use by Blair Bell (1909) who injected extracts of the posterior pituitary into two pregnant women delivering by Cesarean section and noted that upon treatment the uterus contracted into a "blanched ball." Stimulated by this observation, pituitary extracts were soon utilized to treat the life threatening condition of postpartum hemorrhage (Foges and Hofstatter, 1910; Heller, 1970) and later, smaller doses were used to induce labor (Hofbauer, 1911). Consequently, the biological activities of oxytocin were appreciated long before the structure of this peptide hormone was discovered by Du Vigneaud et al. (1953).

Structural characteristics of oxytocin

Oxytocin is one of two hormones that are the primary secretory products of the posterior pituitary. Oxytocin and arginine vasopressin (AVP) are both nonapeptide hormones with a disulfide bond connecting cysteine residues at positions 1 and 6 giving a ring structure that is essential for biological activity (Samson, 1988).

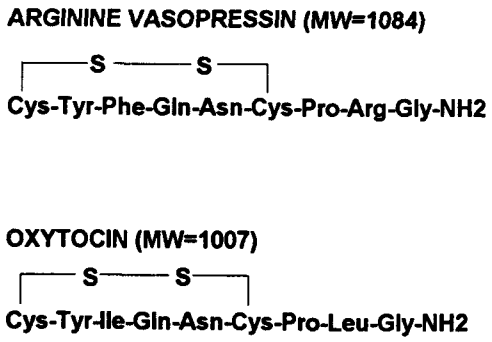

Fig. 1 Primary structure and molecular weights of arginine vasopressin and oxytocin.

Both hormones are synthesized separately in the hypothalamus as part of a larger precursor molecule that consists of the respective hormone and a carrier protein called neurophysin (MW≈10,000). It is probable that these proteins are derived from a common ancestral hormone in the cyclostomes approximately 4 million years ago (Acher, 1979). However, now it is apparent that oxytocin and AVP arise from different genes, each with its own associated neurophysin. Neurophysin has no known biological activity, but it may play a role in protecting oxytocin and AVP from intracellular degradation. The neurophysins are proteolytically cleaved from their respective hormones in secretory granules during axonal transport from the cell bodies in the hypothalamus to the nerve terminals. Both neurophysin and its associated hormone are released into and stored in the posterior pituitary from where they are released into the general circulation.

Although chemically related, oxytocin and vasopressin have adopted quite different physiological functions in the mammal. The stimulation of myometrial contraction in the uterus and milk ejection is attributed to oxytocin, while vasopressin stimulates antidiuretic activities.

Oxytocin gene structure

The bovine cDNA and the rat gene for vasopressin were elucidated before the oxytocin gene (Land et al., 1982, Schmale et al., 1983). Because of homology between the neurophysins associated with these neurohormones, DNA probes from the rat vasopressin gene were used to isolate the oxytocin gene from a rat genomic library (Ivell and Richter, 1984b). Sequence analysis revealed that, like vasopressin, the OT gene consisted of three exons and two intervening introns (Fig. 2).

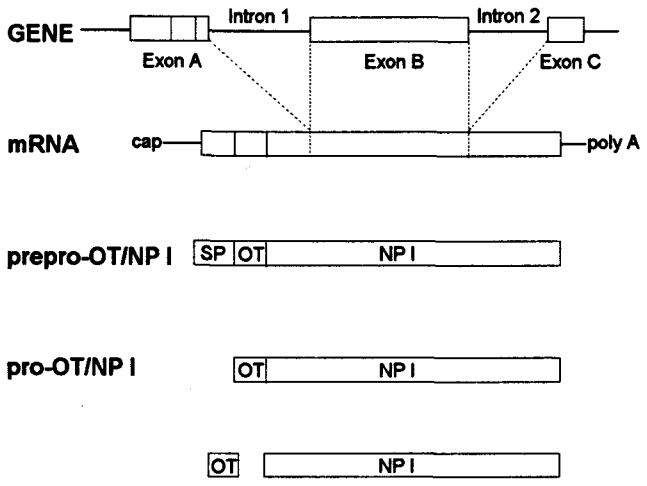

Fig. 2 Gene, messenger RNA (mRNA), prepro-hormone, prohormone and mature hormones for oxytocin (OT) and neurophysin I (NP I). SP=signal peptide. Adapted from Ivell and Richter, 1985.

The first exon codes for a 19 amino acid signal peptide that directs the polypeptide to the endoplasmic reticulum and the secretory pathway. The signal peptide is cleaved off co-translationally as it enters the endoplasmic reticulum. Immediately following the signal peptide, the gene codes for the nine amino acids of the oxytocin hormone. In addition, the 3' end of the first exon codes for the first nine amino acids of neurophysin. A three amino acid sequence of Gly-Lys-Arg in the prepro-hormone separates OT from the first nine

amino acids of neurophysin. The two basic amino acids are a consensus cleavage signal common in many polyprotein precursors. The glycine appears to be involved in the amidation of the preceding amino acid on the oxytocin molecule (Bradbury et al., 1982).

The second exon encodes the constant region of neurophysin. This region is highly conserved between species and between oxytocin and vasopressin. This high degree of homology suggests that neurophysin may have some important function besides acting simply as a carrier molecule for OT during transport in the granules. The third exon encodes the C-terminal, variable region of neurophysin (reviewed by Ivell and Richter, 1985).

Oxytocin in peripheral tissues

Neurons with the capacity to synthesize oxytocin are separate and distinct from those that synthesize vasopressin (Dierickx and Vandesande, 1979; Sofroniev, 1983). However, both types of neurons are concentrated in two distinct areas of the hypothalamus, the paraventricular nucleus and the supraoptic nucleus (Du Vigneaud, 1954; Dierickx and Vandesande, 1979; Sofroniev, 1983). Unlike vasopressin neurons, oxytocin cell bodies appear to be absent from extra-hypothalamic regions of the brain (Sofroniev, 1985).

Oxytocin containing cells have also been identified in several different tissues outside the brain using high performance liquid chromatography (HPLC), immunohistochemistry or radioimmunoassay. These tissues include the adrenal medulla (Ang and Jenkins, 1984; Nicholson et al., 1984), testicular interstitial cells (Nicholson et al., 1984; Guldenaar and Pickering, 1985), the corpus luteum (Wathes and Swann, 1982; Flint and Sheldrick, 1982; Fields et al., 1983) the placenta (Fields et al., 1983; Makino et al., 1983) and small cell carcinomas of the lung (Maurer et al., 1983). However, except for the corpus luteum, there is no evidence that the tissues where oxytocin has been

localized are also the site of synthesis. A discussion of sequence analysis of the oxytocin peptide and mRNA will follow (Ivell and Richter, 1984a).

Oxytocin in the corpus luteum

As early as 1910, Ott and Scott found that an aqueous extract of the corpus luteum increased milk flow. These observations were confirmed in several experiments in which acidic extracts of nonpregnant ovine and bovine corpora lutea stimulated contraction in rat and mouse uterine strip bioassays, respectively (Fields et al., 1980; Wathes and Swann, 1982; Fields et al., 1983). In addition, the immunological data described in the previous section demonstrated that the corpus luteum contains oxytocin. However, these experiments did not demonstrate whether the hormone was synthesized locally or sequestered from the general circulation to be released later in response to some specific stimulation. Dot blot analysis was used to demonstrate that the oxytocin gene is indeed highly transcribed in the corpus luteum with the highest expression occurring early in the cycle. In fact, the active corpus luteum produces approximately 250 times more oxytocin mRNA than a single hypothalamus on a per organ basis (Ivell and Richter, 1984a).

Comparison of luteal and hypothalamic oxytocin

In an elegant series of experiments, Ivell and Richter (1984a) elucidated the biosynthesis and sequence of OT mRNA in the bovine corpus luteum and compared these results to those known for the hypothalamus. Restriction analysis and Southern blot of calf cerebellum DNA revealed that calf oxytocin synthesis must result from the activity of only a single oxytocin gene. However, one cannot overlook the possibility that tissue specific alternative splicing may yield a luteal oxytocin mRNA that is not identical with the

hypothalamic transcript. Therefore, Ivell and Richter (1984a) cloned poly A^+ RNA from the cow corpus luteum into the plasmid pUC9 and screened with an oxytocin-specific probe for those colonies with a positive signal for the OT mRNA. The longest insert was sequenced and was determined to be identical to that reported for the hypothalamic cDNA (Land et al., 1983) and the bovine OT gene (Ruppert et al., 1984). Northern blot analysis was also performed to further characterize the OT mRNA from the corpus luteum. Using probes derived from different parts of the cDNA, it was determined that luteal OT mRNA migrated as a single band of about 630 nucleotides. This is somewhat smaller than hypothalamic OT mRNA, which is about 690 bases in length. It was hypothesized that this small difference in length was a consequence of differential polyadenylation. This hypothesis was substantiated by incubating OT mRNA from luteal or hypothalamic origin with oligo(dT) and RNase H, a treatment that causes the preferential removal of the poly A tail. This treatment reduced the size of the transcript from both tissues to approximately 590 bases. Therefore, the difference in the length of the OT mRNA between the hypothalamus and the corpus luteum can be attributed to the different lengths of the poly A tail.

Finally, to demonstrate that the selected luteal OT mRNA could code for the appropriate OT precursor, an *in vitro* translation system using rabbit reticulocyte lysates was used. Antibodies raised against oxytocin-neurophysin specifically immunoprecipitated a polypeptide product of the translation system with a molecular weight of 16,500, similar to that coded by the hypothalamic mRNA (Ivell and Richter, 1984a).

Coincident experiments performed by Swann et al. (1984) also demonstrated that the corpus luteum synthesized oxytocin locally. Dispersed cell cultures of bovine and ovine corpora lutea were incubated with [^{35}S]-cysteine to follow the incorporation of the isotope into oxytocin and neurophysin. After 12 hours incubation, acid extraction of cultures, neurophysin-sepharose affinity chromatography and HPLC, a single 14 kD band was observed on sodium dodecyl sulphate (SDS)-gels. The protein in this band was

immunoprecipitated with antibodies against anti-oxytocin and anti-neurophysin and migrated in an identical fashion to rat hypothalamic oxytocin-neurophysin. Furthermore, treatment of cells with the protein synthesis inhibitor, cyclohexamide, blocked the incorporation of [^{35}S]-cysteine into oxytocin and neurophysin in the CL.

Regulation of oxytocin synthesis from the corpus luteum

Oxytocin gene expression has been measured in the corpora lutea of cattle and sheep. In both species mRNA levels were highest early in the estrous cycle and decline precipitously until mid-cycle (Fehr et al., 1987; Jones and Flint, 1988; Ivell et al., 1990). It is suggested that a marked decrease in transcription of the OT gene occurs early in the luteal phase of the cycle and thereafter mRNA degradation occurs at a constant rate. This single large pulse of oxytocin mRNA is correlated with ovulation and early luteinization and is in marked contrast to OT gene expression in the hypothalamus, which exhibits no variation with the estrous cycle. These results emphasize the independent nature of transcriptional control in these two tissues. This control must reside in the local cellular environment and result from different cell-specific receptor systems (Ivell et al., 1985).

Although transcription of the oxytocin gene coincides with the time of ovulation, the factors triggering this expression are not known. The dramatic increase in OT gene expression on day 1 of the estrous cycle is preceded by a smaller peak of OT mRNA in some preovulatory follicles on the day of estrus. Therefore, it is clear that ovulation alone cannot be the sole stimulator of oxytocin gene expression. Evaluation of the hormonal profile around the time of ovulation reveals that there are increased systemic levels of LH, FSH and estrogen. Voss and Fortune (1992) have demonstrated that there is significantly more OT mRNA in granulosa cells isolated after the LH/FSH surge than in those isolated before this surge of gonadotropins. It is conceivable that the LH/FSH surge mediates its effect on the OT gene via cAMP and the cAMP responsive element (CRE). In this regard,

the 3000 bp 5' non-coding region of the bovine OT gene contains consensus regulatory elements for cAMP as well as for estrogen, glucocorticoids, thyroid hormone and phorbol esters (reviewed by Wathes and Denning-Kendall, 1992). However, the physiological relevance of these sites has not been established except for estrogen. An indirect effect of the gonadotropins cannot be ruled out.

Estrogen response elements (EREs) as well as estrogen responsiveness have been demonstrated for the 5'-flanking sequences of the human and rat OT genes. Evaluation of 5' deletion mutants of the rat upstream region linked to the luciferase gene revealed two regions (-172 to -149 and -148 to +16) that were associated with an estrogen-induced 15-fold increase in activity of the homologous promoter. The former site contains an imperfect estrogen response element that differs by one nucleotide from the consensus sequence. Unlike the rat OT gene, the bovine gene is not responsive to estrogen. The rat ERE differs by three nucleotides from the corresponding motif in the bovine gene (Adan et al., 1991). Instead, this region in the bovine gene shows an almost perfect homology with the chicken ovalbumin upstream promoter (COUP) orphan receptor binding site found in the chicken ovalbumin gene (Walther et al., 1991). This region was shown to bind two nuclear proteins, one of which was similar to the COUP protein. This protein was present throughout the luteal phase and inhibited transcription of the OT gene. In the early luteal phase another nuclear protein of unknown identity was produced by the corpus luteum and has been proposed to displace the COUP-related factor and activate OT gene transcription.

Secretion of oxytocin from the bovine corpus luteum

Oxytocin synthesis in the ovary follows a similar pathway to what has been reported for the hypothalamus. The growing polypeptide chain is taken up into the endoplasmic reticulum and the signal peptide removed before passage into the Golgi

apparatus. The Golgi apparatus packages the pro-oxytocin/neurophysin into secretory granules where it is stored and further processed to oxytocin (Ivell, 1986). Oxytocin release must occur via a regulated rather than a constitutive pathway because OT is packaged into granules instead of released immediately upon translation. Several factors have been implicated in the control of oxytocin release from the bovine corpus luteum.

Oxytocin is released from the posterior pituitary in response to an action potential that causes an increase in intracellular Ca^{2+} concentration (Nordmann, 1983). Hirst et al. (1986) demonstrated that calcium is also involved in the secretion of OT from the ovine corpus luteum. Incubation of ovine luteal slices in calcium deficient medium caused a reduction in secretion of OT. Addition of 100 mM potassium (a concentration that causes calcium influx) or the calcium ionophore, A23187, to the medium caused an increase in oxytocin secretion. These results are consistent with those reported by Baum and Rosenberg (1987) and Cosola-Smith et al. (1990), who demonstrated that incubation of rat dispersed luteal cells or bovine luteal tissue slices, respectively, with A23187 caused a significant increase in OT secretion. Unlike the posterior pituitary, the corpus luteum does not generate an action potential that would lead to calcium influx. However, intracellular calcium concentrations may be increased in this tissue by the generation of inositol polyphosphates that open calcium channels from both intracellular and extracellular stores (Gallacher, 1988).

Treatment of large luteal cells with prostaglandin $F_{2\alpha}$ causes a dramatic and sustained increase in intracellular Ca^{2+} that is derived from extracellular stores (Hansel et al., 1991; Wiltbank et al., 1991). This increase in intracellular calcium may bring about the release of OT from the corpus luteum. Indeed, prostaglandin $F_{2\alpha}$ has been shown to stimulate luteal OT secretion in the cow and the sheep. In addition to causing the influx of calcium from extracellular stores, $PGF_{2\alpha}$ causes phosphoinositide turnover with resultant increases in inositol 1,4,5-trisphosphate (IP_3), which mobilizes calcium from intracellular stores (Leung et al., 1986; Davis et al., 1987).

Several other second messengers besides Ca^{2+} have been implicated in the regulation of OT secretion from the large cells of the corpus luteum. In this regard arachidonic acid, which is liberated from membrane phospholipids by the action of phospholipase A_2 (PLA_2), has been shown to stimulate the release of oxytocin from ovine luteal tissue slices (Hirst et al., 1988). This activity was not affected by the addition of cyclooxygenase inhibitors to the medium suggesting that the action of arachidonic acid was direct and not the result of its conversion to prostaglandins.

Hirst et al. (1988) also demonstrated that phospholipase C (PLC) stimulates the release of oxytocin from ovine luteal tissue slices. Phospholipase C hydrolyzes membrane phospholipids generating diacylglycerol (DAG) and releasing the phosphate head group (e.g., IP_3). These two second messengers of phospholipid origin are known activators of protein kinase C (PKC), DAG acting directly and IP_3 by causing the release of intracellular calcium. Protein kinase C has been implicated in exocytotic processes in several other tissues that will be discussed subsequently (Nishizuka, 1984). Therefore, it is proposed that PKC may be involved in the secretion of OT from the corpus luteum.

Cosola-Smith et al. (1990) examined the involvement of PKC in the release of OT from the bovine corpus luteum. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) was used as a specific activator of protein kinase C. Results from this experiment revealed that treatment of day 8 bovine luteal tissue slices with TPA caused a significant increase in OT release, thus supporting the concept that PKC is involved in this exocytotic process. Protein kinase C has also been implicated in the regulation of OT secretion from the ovine corpus luteum (Hirst et al., 1988). The potential physiological role of this second messenger in the secretion of oxytocin from the bovine corpus luteum will be discussed in depth in a subsequent section.

β-Adrenergic stimulation may also regulate the secretion of OT from the corpus luteum, although the mechanism of action is not clear. Injection of noradrenaline into the aorta abdominalis stimulates the secretion of ovarian oxytocin and progesterone (Kotwica

et al., 1991). However, the same dose of this catecholamine injected into the jugular vein has no effect, indicating that the adrenergic stimulus is acting locally on the ovary. The stimulatory effect of noradrenaline is mediated through β- and not α-adrenergic receptors as demonstrated by Skarzynski and Kotwica (1993). Noradrenaline-induced oxytocin secretion was inhibited by the β-blocker propranolol, but not the α-blocker, phentolamine. Mammalian ovaries are richly supplied with adrenergic nerves, which are the local source of this biogenic amine (Stefenson et al., 1984). It has been suggested that it is the small cells of the corpus luteum and not the large cells that contain β-adrenergic receptors (Skarzynski and Kotwica, 1993). If this is true, cell-to-cell communication would be required to pass the adrenergic signal from the small to the oxytocin laden large cells of the corpus luteum. However, it should be noted that receptor concentrations on the two cell types were not measured in these experiments. Progesterone production was used as the end point to determine cell responsiveness to the adrenergic stimulus.

Estradiol-17β has been shown to cause the release of oxytocin from cultured bovine granulosa cells (Voss and Fortune, 1993). This steroid exhibited a biphasic effect on oxytocin secretion; low concentrations caused a significant increase in OT production, while high concentrations of estradiol inhibited the secretion of this nonapeptide. There is no evidence that estradiol-17β or any other steroid has an effect on oxytocin secretion from the corpus luteum.

Prostaglandin $F_{2\alpha}$ has consistently been shown to stimulate the secretion of oxytocin from the ruminant corpus luteum *in vivo* (Schallenberger et al., 1984; Walters et al., 1984; Lamsa et al., 1989; Flint et al., 1990). However, the effect of $PGF_{2\alpha}$ on luteal oxytocin secretion has been difficult to substantiate in *in vitro* experiments. Abdelgadir et al. (1987) showed a dose response effect of $PGF_{2\alpha}$ on oxytocin secretion from day 8 bovine luteal tissue slices. Jarry et al. (1992) used a reverse haemolytic plaque assay to measure oxytocin release from cultured bovine luteal cells and found that treatment with $PGF_{2\alpha}$ caused an increase in plaque size, indicative of increased oxytocin secretion.

Wathes and Denning-Kendall (1992) reported unpublished results indicating that $PGF_{2\alpha}$ caused increased oxytocin secretion from luteinized ovine granulosa cells. In contrast, several laboratories have been unable to demonstrate an acute effect of $PGF_{2\alpha}$ on luteal oxytocin release *in vitro*; including McArdle and Holtorf (1989) using bovine luteal cells, Hirst et al. (1986, 1988) using ovine luteal slices and Wathes et al. (1988) using bovine luteal pieces in a perifusion system. It is conceivable that samples in these *in vitro* experiments had an inconsistent number of large cells, thus leading to the dichotomy of responses to $PGF_{2\alpha}$. In this regard, please note that the luteinized granulosa cells used by Wathes and Denning-Kendall should represent a relatively pure population of large luteal cells and they did respond to $PGF_{2\alpha}$. Several other parameters may have affected the *in vitro* response of luteal tissue to $PGF_{2\alpha}$, including time from slaughter to tissue collection, physical manipulation of the tissue during surgical procedures and medium and temperature differences for the transport of tissues to the laboratory.

Regulation of oxytocin secretion from the corpus luteum by $PGF_{2\alpha}$ is further complicated by the reports of Heap et al. (1989) who demonstrated that infusion of $PGF_{2\alpha}$ into the uterine lymphatics *in vivo* caused a significant release of luteal OT from both ovaries. It is considered unlikely that the prostaglandin could have reached the contralateral ovary in a concentration sufficient to evoke a response. However, it is possible that physical manipulation of the opposite ovary would cause a local release of $PGF_{2\alpha}$ that would stimulate OT release. It was proposed that $PGF_{2\alpha}$ may be mediating its effect via a neural mechanism. As stated above, adrenergic innervation of the ovary may mediate this effect. However, α- and β-adrenergic blockers could not block the secretory effect of $PGF_{2\alpha}$, indicating that $PGF_{2\alpha}$ exerts its effect by a different mechanism (Kotwica et al., 1991).

Based on the *in vivo* data and a subset of the *in vitro* data, it is proposed that $PGF_{2\alpha}$ does cause a release of oxytocin from the ruminant corpus luteum and this effect is

mediated by increased intracellular calcium concentrations and activation of protein kinase C.

Actions of ovarian oxytocin (luteolysis)

Oxytocin has been implicated as a luteolytic hormone in cows, sheep, does, Rhesus monkeys and humans. The luteolytic action of oxytocin was first described in cows by Armstrong and Hansel (1959). Daily administration of natural or synthetic oxytocin to nonlactating dairy heifers during the first week of the estrous cycle shortened the cycle from the usual 21 days to 8-12 days. However, treatment at later times in the cycle did not affect estrous cycle length (Hansel and Wagner, 1960). Corpora lutea formed during the oxytocin treatment did not attain the expected size and had a decreased number of luteal cells and an increased amount of connective tissue. Animals treated daily with progesterone in addition to oxytocin did not return to estrus until 4 days after the withdrawal of progesterone. Therefore, it was suggested that treatment with OT caused decreased luteal function which was manifested by a reduction in progesterone, thus, permitting the pituitary to secrete estrous-inducing gonadotropins. Oxytocin treatment did not effect luteal function in hysterectomized animals. This observation implicated the uterus as a mediator of the luteolytic effect of oxytocin. Treatment of intact goats with exogenous oxytocin early in the cycle also caused reduced estrous cycle length (Cooke and Knifton, 1981; Cooke and Homeida, 1982).

Administration of exogenous oxytocin does not affect estrous cycle length in sheep, although it does cause a slight reduction in systemic progesterone (Milne, 1963; Hatjiminaoglou et al., 1979; Cooke and Homeida, 1982). However, active immunization against oxytocin prolongs the luteal phase of the cycle in ewes and therefore implicates OT as a luteolytic agent in this species (Sheldrick et al., 1980; Schams et al., 1982).

Injection of oxytocin into the ovarian artery of the Rhesus monkey caused a significant decrease in progesterone secretion and reduced the length of the menstrual cycle (Aulleta et al., 1984). Similarly, treatment of human dispersed luteal cells with oxytocin inhibited both basal and gonadotropin-induced secretion of progesterone (Tan et al., 1982).

Although the early research from Hansel's laboratory indicated that the luteolytic effect of OT was mediated by the uterus, the mechanism of this uterine involvement was not known. McCracken et al. (1972) demonstrated that $PGF_{2\alpha}$ of uterine origin was the principal luteolysin in sheep and questions regarding the control of secretion of this prostanoid began to arise. Thus, oxytocin may be exhibiting its uterine dependent luteolytic effect by stimulating the release of $PGF_{2\alpha}$. Several investigators have demonstrated that oxytocin causes the release of prostaglandins from the uterus *in vivo* (Sharma and Fitzpatrick, 1974; Mitchel et al., 1975; Roberts et al., 1975). The uterine myometrium is a known target of oxytocin (Soloff et al., 1974), but the primary source of uterine prostaglandins in several species is the endometrium (Pickles, 1967; Wilson et al., 1972; Williams, 1973; Downie et al., 1974; Levitt et al., 1975). It has been proposed that OT-induced mechanical changes in the uterine wall (i.e. myometrial contraction) could lead indirectly to the release of prostaglandins (Poyser et al., 1971; Kloeck and Jung, 1973). However, infusion of oxytocin into the uterine artery of the ewe toward the end of the estrous cycle fails to alter myometrial activity, but still causes the release of $PGF_{2\alpha}$. Therefore, Roberts et al. (1976) examined the uterine endometrium for specific OT binding sites and determined the influence of OT on prostaglandin secretion from this tissue.

Examination of ovine endometrium tissues revealed that the endometrium is a specific target for oxytocin (Roberts et al., 1976). Furthermore, binding of OT to its endometrial receptor stimulates the release of $PGF_{2\alpha}$. Secretion of this prostanoid from ovine endometrium was minimal on day 5 of the estrous cycle and maximal on days 13 and

15, which coincides with the time of luteal regression in this species. Treatment of endometrial tissue with OT potentiated the release of $PGF_{2\alpha}$ from day 13, 15 and 0 of the estrous cycle, but not from tissue collected on day 3 or 5. Based on the stage of cycle dependence of OT-induced $PGF_{2\alpha}$ release from this tissue, these researchers suggested that ovarian steroids affect endometrial prostaglandin synthesis by regulating OT receptor levels. This hypothesis is supported by Soloff (1975), who demonstrated that treatment of ovariectomized rats with estrogen leads to enhanced OT binding by the uterus and oviducts. Collectively, these data provide strong evidence that $PGF_{2\alpha}$ mediates the uterine dependent luteolytic effect of oxytocin.

$PGF_{2\alpha}$ is the Principal Luteolytic Factor in Several Mammalian Species

The importance of the uterus in regression of the corpus luteum was initially described by Loeb (1923) who showed that normal regression of the corpus luteum did not occur in hysterectomized guinea pigs. Similar effects of hysterectomy have been reported for several mammalian species (Anderson et al., 1969). Subsequent experiments involving transplantation of the uterus into the neck of sheep, demonstrated that the uterus and ovary must be in close proximity to each other for normal luteal regression to occur (Harrison et al., 1968; McCracken et al., 1971). Using a series of cross-cannulation experiments between sheep at different stages of the estrous cycle, McCracken et al. (1972) demonstrated that some unknown product secreted into the uterine vein near the end of the estrous cycle was capable of causing luteal regression. Pharriss and Wyngarden (1969) had previously suggested that because $PGF_{2\alpha}$ was an abundant product of the uterus, this compound might fulfill the role of the uterine luteolysin. These researchers demonstrated that a large subcutaneous injection of $PGF_{2\alpha}$ into rats caused a significant shortening of the estrous cycle, but attributed this effect to the ability of $PGF_{2\alpha}$ to inhibit ovarian blood flow. Subsequently, McCracken et al. (1972) reported that injection of low

doses of $PGF_{2\alpha}$ into the ovarian artery of ewes caused premature regression of the corpus luteum and the induction of a new estrous cycle. However, systemic infusions of the free acid form of this prostanoid were not effective, which can be partially explained by the dilution effect and partly by the fact that $PGF_{2\alpha}$ is known to be degraded rapidly with one pass through the lungs in some mammalian species (Piper et al., 1970).

Collectively, these experiments explained why the luteolytic effect of the uterus must be local, but did not explain how uterine $PGF_{2\alpha}$ reached the ovary without entering the general circulation. In this regard, McCracken et al. (1972), based on personal communications with William Hansel, suggested that $PGF_{2\alpha}$ may diffuse from the uterine vein into the ovarian artery via a counter-current flow mechanism. This is a reasonable suggestion because most of the ovarian artery is in close apposition to the utero-ovarian vein. To test this counter-current hypothesis, McCracken et al. (1972) infused ^{3}H-$PGF_{2\alpha}$ into the uterine vein of sheep on day 14 of the cycle. Blood samples were collected via catheter from the ovarian artery just before it entered the ovary and from the adjacent iliac artery. The amount of labeled $PGF_{2\alpha}$ found in the ovarian artery increased markedly as the infusion progressed, while only a small increase in the radioactive prostanoid was observed in the peripheral blood sample. Thus, these data indicated that a transfer of ^{3}H-$PGF_{2\alpha}$ from the uterine vein to the ovarian artery had occurred. Subsequent experiments have demonstrated that $PGF_{2\alpha}$ is indeed the luteolytic agent in several mammalian species including cattle (Liehr et al., 1972; Rowson et al., 1972; Louis et al., 1974), sheep (McCracken et al., 1970; Barrett et al., 1971; Thorburn and Nicol, 1971), guinea pigs (Chaichareon et al., 1974), goats (Ott et al., 1980), horses (Douglas and Ginther, 1972; Allen and Rowson, 1973; Noden et al., 1974), pseudopregnant mice (Bartke et al., 1972), hamsters (Harris and Murphy, 1981), rabbits (Scott and Rennie, 1970; Gutknecht et al., 1972) and swine (Diehl and Day, 1973; Gleeson, 1974; Douglas and Ginther, 1975; Hallford et al., 1975).

Mechanism of $PGF_{2\alpha}$-induced regression of the corpus luteum

A number of different mechanisms have been proposed to explain the luteolytic effect of prostaglandin $F_{2\alpha}$. These mechanisms can be separated into two categories: 1) one or more direct actions on luteal cells and 2) a decreased blood flow to the corpus luteum.

Prostaglandin $F_{2\alpha}$ has several direct effects on luteal function. Several investigators have demonstrated that $PGF_{2\alpha}$ is antagonistic to the action of gonadotropins. In this regard $PGF_{2\alpha}$ has both an acute and a prolonged effect. Initially the prostanoid blocks LH-stimulated cAMP production (Lahav et al., 1976). In the rat, this acute effect on gonadotropin-induced cAMP accumulation is not mediated through interference with LH binding (Thomas et al., 1978). However, long term exposure to $PGF_{2\alpha}$ (12-24 hours) caused a significant reduction in LH receptor concentrations in the rat corpus luteum (Behrman et al., 1979). Agudo et al. (1984) demonstrated that *in vivo* treatment of ewes with $PGF_{2\alpha}$ caused a decrease in adenylate cyclase activity and an increase in phosphodiesterase activity. Taken together, these effects would cause a decrease in cellular cAMP and may be the early event that leads to decreased progesterone production by the regressing CL in ewes. In addition to the rat and the ewe, $PGF_{2\alpha}$ has been shown to inhibit LH-stimulated progesterone production in the cow (Marsh, 1971), woman (Hamberger et al., 1979), nonhuman primate (Stouffer et al., 1979) and sow (Ritzhaupt et al., 1986). However, it should be noted that luteal function is not affected by hysterectomy in women or monkeys (Castracane et al., 1979). Intraluteal injection of physiological concentrations of $PGF_{2\alpha}$ into the corpus luteum of women (Wentz and Jones, 1973; Korda et al., 1975) and Rhesus monkeys (Auletta et al., 1984; Zelinski-Wooten and Stouffer, 1990) causes premature luteolysis. The human corpus luteum produces and contains binding sites for $PGF_{2\alpha}$ (Powell et al., 1974; Rao et al., 1977; Tanaka et al., 1983) and luteal concentrations of $PGF_{2\alpha}$ increase during the late luteal

phase of the estrous cycle (Shutt et al., 1976; Patwardhan and Lanthier, 1980; Vijayakumar and Walters, 1983). An intra-ovarian role for oxytocin in the regression of the primate corpus luteum has yet to be established.

The observed effect of $PGF_{2\alpha}$ on LH-stimulated progesterone production by the corpus luteum is interesting because the majority of LH receptors are on small luteal cells, while the majority of $PGF_{2\alpha}$ receptors are located on large luteal cells (Fitz et al., 1982). This suggests that there must be some cell-to-cell communication between small and large luteal cells during luteolysis. Indeed, Rodgers et al. (1985) have demonstrated that $PGF_{2\alpha}$ has no effect on LH-stimulated progesterone production from a pure small cell population, but significantly inhibits this activity in small cells contaminated by large cells. Furthermore, plasma membranes of the regressing corpus luteum develop gap junctions that may facilitate the intercellular transport of biochemicals in the intact corpus luteum (Niswender and Nett, 1988).

Cholesterol is the precursor for steroid biosynthesis in steroidogenic tissues. Cultured bovine luteal cells can utilize low density lipoprotein (LDL), high density lipoprotein (HDL) or can synthesize cholesterol *de novo* from acetate. Treatment of cultured bovine luteal cells with either HDL or LDL enhances progesterone production (Pate and Condon, 1989). However, this steroidogenic effect of lipoproteins is inhibited by $PGF_{2\alpha}$. In addition $PGF_{2\alpha}$ inhibited *de novo* cholesterol synthesis and decreased the conversion of cholesterol to progesterone in this tissue. The rate limiting step in steroid biosynthesis is the cleavage of the cholesterol side chain, brought about by the action of the mitochondrial cytochrome P-450 enzyme complex. Pate and Condon (1989) suggested that $PGF_{2\alpha}$ inhibits the action of this enzyme that catalyzes the conversion of cholesterol to pregnenolone, thereby preventing the utilization of lipoprotein-derived cholesterol. It has also been suggested that treatment of the ovine corpus luteum with $PGF_{2\alpha}$ causes a reduction in 3β-hydroxysteroid dehydrogenase activity, which catalyzes

the conversion of pregnenolone to progesterone (Hoppen and Findlay, 1976; McClellan et al., 1977).

Degenerative changes in the plasma membranes of regressing corpora lutea have been correlated with increased phospholipase A_2 (PLA_2) activity (Riley and Carlson, 1986). Phospholipase A_2 can be activated by increased intracellular calcium concentrations possibly brought about by the action of $PGF_{2\alpha}$. Arachidonic acid is released by the action of PLA_2 from membrane phospholipids and metabolism of this eicosanoid via the cyclooxygenase pathway can lead to luteal $PGF_{2\alpha}$ production. Rothchild (1981) proposed that prostaglandin $F_{2\alpha}$ of uterine or ovarian origin could stimulate its own production in luteal tissue, thus contributing to the completion of luteolysis in a paracrine fashion.

It has also been suggested that arachidonic acid metabolism may lead to the accumulation of lipoxygenase products that are involved in luteal regression. In this regard Hansel and Dowd (1986) suggested that 5-hydroxyeicosatetraenoic acid (5-HETE) may have luteolytic function in cattle. This suggestion is supported by the work of Orwig et al. (1992, see appendix) who demonstrated that 12,13-dihydroxyeicosapentaenoic acid (12,13-diHEPE), a polyunsaturated product of the lipoxygenase pathway isolated from red algae, interferes with LH-stimulated progesterone production from ovine luteal tissue slices. These authors found that the majority of ^{14}C-labeled 12,13-diHEPE was associated with the plasma membrane and suggested that incorporation of this polyunsaturated fatty acid may lead to a decrease in membrane fluidity. Such a change in membrane characteristics could hinder LH receptor-associated G-protein mobility and subsequent activation of adenylate cyclase (Shechter et al., 1974). This hypothesis is supported by the observation that a liquid to gel phase transition occurs in rat luteal cell membranes during natural luteal regression (Buhr et al., 1979; Carlson et al., 1984). This change in membrane characteristics has also been observed in $PGF_{2\alpha}$-induced luteal regression in rats (Sawada and Carlson, 1991).

Finally, it should be noted that $PGF_{2\alpha}$ has a direct cytotoxic effect on luteal tissue in the ewe. Specifically, Silvia et al. (1984) have demonstrated that $PGF_{2\alpha}$ is cytotoxic to large luteal cells *in vitro* and that this effect was reversed by treatment with the luteotropin, PGE_2.

Injection of a luteolytic dose of $PGF_{2\alpha}$ into rats does not alter luteal blood flow (Behrman et al., 1979). However, in ewes, the first effect of $PGF_{2\alpha}$ appears to be a decrease in luteal blood flow and this effect is correlated with decreased progesterone concentrations in the peripheral circulation (Niswender et al., 1976). However, no cause and effect relationship has been established between blood flow and progesterone concentrations. The decrease in luteal blood flow probably results from the known vasoconstrictor property of $PGF_{2\alpha}$ (Pharris and Wyngarden, 1969). Effect of $PGF_{2\alpha}$ on ovarian blood flow as a cause of luteal regression is controversial and may be species specific.

Prostaglandin $F_{2\alpha}$/oxytocin snowball effect

Based on the reports cited above, it is clear that both ovarian oxytocin and uterine $PGF_{2\alpha}$ are involved in regulating the regression of the corpus luteum, at least in some mammalian species. It is also evident that $PGF_{2\alpha}$ and OT are capable of regulating each others release from the uterus and ovary, respectively. Furthermore, it has been demonstrated that during luteolysis in ewes, $PGF_{2\alpha}$ is released from the uterus as a series of pulses lasting one hour each, with a periodicity of about 6 hours (McCracken, 1984). Pulsatile release of $PGF_{2\alpha}$ from the uterus has also been observed in the cow, sow and mare (McCracken et al., 1984). In ewes it was demonstrated that five physiological pulses (one-hour each) of prostaglandin $F_{2\alpha}$ infused into the ovarian artery over a period of 25-30 hours was the minimal requirement for luteal regression (Schramm et al., 1983). Based on these observations, McCracken (1984) proposed a positive feedback model between

uterine prostaglandins and ovarian OT to explain the mechanism of luteal regression and the pulsatile release of $PGF_{2\alpha}$ in sheep. This model also requires the indirect involvement of ovarian progesterone and estradiol. In ewes, estradiol stimulates the production of endometrial OT receptors in progesterone primed tissue, a process that requires approximately 6 hours (McCracken, 1980; Zhang et al., 1992). Activation of these receptors by oxytocin immediately evokes the release of $PGF_{2\alpha}$. However, during the luteal phase of the estrous cycle, high progesterone blocks the nuclear localization of estradiol receptors in the uterine endometrium and thereby, the ability of estradiol to stimulate the synthesis of OT receptors (Leavitt et al., 1985). After long exposure to progesterone, this steroid causes the down-regulation of its own receptors in the uterus (Milgrom et al., 1973), thus, estradiol is again able to stimulate the production of OT receptors. In addition to blocking uterine responsiveness to estradiol, progesterone promotes the accumulation of fatty acid precursors to $PGF_{2\alpha}$ (Brinsfield and Hawk, 1973) and increases prostaglandin H synthase activity in the endometrium of the cow (Basu and Kindahl, 1987; Gross et al., 1988) and sheep (Huslig et al., 1979; Salamonsen and Findlay, 1990). Consequently, when the inhibitory effect of progesterone diminishes at the end of the cycle, endogenous OT from the posterior pituitary or the CL stimulates a release of $PGF_{2\alpha}$ 100 times greater than early in the cycle, before progesterone exposure. This explosive release of $PGF_{2\alpha}$ from the uterus then initiates luteolysis in the ovary via the counter-current transfer mechanism. Initially, oxytocin released from the CL by the action of $PGF_{2\alpha}$ may reinforce the secretion of the prostanoid from the uterus, but oxytocin also rapidly causes the down-regulation of its own receptor. Subsequent pulses of $PGF_{2\alpha}$ will occur every 6 hours because that is the time required for estradiol to regenerate the OT receptors in the endometrium (McCracken, 1980).

Various components of the $PGF_{2\alpha}$/OT positive feedback loop have been confirmed in other laboratories. In ruminants it has been demonstrated that pulses of OT occur concurrently with $PGF_{2\alpha}$ during luteal regression (Fairclough et al., 1980; Webb et

al., 1981; Flint and Sheldrick, 1983; Vighio and Liptrap, 1986). Most of the oxytocin in these pulses is of luteal and not neurohypophyseal origin (Walters et al., 1984; Hooper et al., 1986; Moore et al., 1986). Flint et al. (1990), in agreement with McCracken, propose that the high concentrations of OT and $PGF_{2\alpha}$ achieved in each pulse are the result of the activation of the positive feedback loop. However, the factor(s) that initiate the loop have not been determined.

It seems to the author of this thesis that the diminishing effect of progesterone on the uterus near the end of the cycle is sufficient to stimulate the positive feedback loop. The increased uterine responsiveness to the OT already in the general circulation would be perceived by the uterus as a pulse of OT and would stimulate the initial burst of $PGF_{2\alpha}$. However, McCracken et al. (1991) recently showed that the changing levels of progesterone and estradiol-17β at the end of a normal estrous cycle, when mimicked in ovariectomized ewes, cause the pulsatile release of OT from the posterior pituitary. It was proposed that this neurohypophyseal OT is the signal that initiates the positive feedback loop.

Subcellular Regulation of Oxytocin Secretion From the Corpus Luteum

Modification of the positive feedback loop involving uterine $PGF_{2\alpha}$ and luteal OT will be required as new information on the topic unfolds. In this regard, there is a paucity of information regarding the subcellular mechanisms that control the release of these two biochemicals from their respective tissues. Upon binding their cell surface receptors, $PGF_{2\alpha}$ and oxytocin both stimulate phosphoinositide hydrolysis with resultant increases in diacylglycerol and intracellular calcium concentration (Leung et al., 1986; Flint et al., 1986; Davis et al., 1987; Wallace et al., 1993). However, the intracellular events that regulate the release of these hormones, beyond the phosphoinositide cascade are not known. Therefore, the focus of the research presented herein is to elucidate the

intracellular signal transduction mechanism that regulates the $PGF_{2\alpha}$-induced release of oxytocin from the bovine corpus luteum.

Protein kinase C

Results of previous research indicate that prostaglandin $F_{2\alpha}$-induced secretion of oxytocin from the bovine corpus luteum may be mediated by the activation of protein kinase C (PKC). It has been demonstrated that $PGF_{2\alpha}$ provokes phosphoinositide hydrolysis with resultant increases in diacylglycerol (DAG) and intracellular Ca^{2+} concentrations (Leung et al., 1986; Davis et al., 1987). Because DAG and Ca^{2+} are known activators of PKC, it is hypothesized that this kinase may regulate the secretion of OT from the large cells of the bovine CL, the source of this nonapeptide hormone. In this regard Cosola-Smith et al. (1990) demonstrated that treatment of bovine luteal tissue slices with 12-O-tetradecanoylphorbol-13-acetate (TPA), a specific activator of PKC, mimicked the effect of $PGF_{2\alpha}$ by inducing OT secretion. Protein kinase C has been shown to play a role in exocytosis in a wide variety of cell types, including several endocrine tissues (Naor et al., 1989; Kikkawa and Nishizuka, 1986; Robinson, 1991).

Protein kinase C is a serine/threonine kinase that exists as a family of multiple subspecies having closely related structures. At least 10 isoforms of this enzyme (α, βI, βII, γ, δ, ε, η, λ, θ and ζ) have been identified (Nishizuka, 1992). These isozymes exhibit slightly different modes of activation, kinetic properties and tissue distribution. The α, βI, βII and γ isozymes have been designated conventional PKCs and are activated by calcium, phospholipids and diacylglycerol (or phorbol ester). The δ, ε, η and θ isozymes, designated novel PKCs, lack the calcium-binding domain found in the cPKCs; however, they are activated by phospholipids and diacylglycerol or phorbol ester. The λ and ζ isozymes are further atypical in that they are not affected by calcium, diacylglycerol or phorbol ester, but are phosphatidylserine-dependent.

In addition to different modes of activation, the various isozymes of protein kinase C have different subcellular localization and substrate specificity, allowing the kinase to participate in a multitude of cellular events. These events include, but are not limited to, lipogenolysis and glucose transport in adipocytes, smooth muscle contraction, steroidogenesis in Leydig cells, glycogenolysis in hepatocytes and hormone secretion from several tissues, including platelets, the adrenal gland, pituitary cells, pancreas and various neurons (Kikkawa and Nishizuka, 1986). However, little is known about the possible function of protein kinase C isozymes in the corpus luteum. Although PKC has been implicated in progesterone production (Wiltbank et al., 1989) and OT secretion (Cosola-Smith et al., 1990) from the ruminant corpus luteum, the actual isozymes present and their mechanism of action in this tissue are not known. In addition, the subcellular localization and potential substrates of this second messenger need to be determined.

Calpains regulate the activity of protein kinase C

Calpains, ubiquitous calcium-activated neutral proteases, may represent an important point of regulation of PKC activity. These cysteine proteases exist in two forms, μ-calpain and m-calpain, which are activated by μ- and mM concentrations of calcium, respectively (Murachi, 1983). It has been demonstrated that calpains hydrolyze membrane-associated PKC molecules to lower molecular weight forms of the enzymes (PKMs) that are constitutively active (Melloni et al., 1986; Nishizuka, 1986). The susceptibility of PKC to hydrolysis by calpain increases significantly under conditions in which PKC is active (Suzuki, 1991). Calmodulin and protein kinase C are recognized as important calcium-dependent cellular second messengers. Because calpains are intracellular proteases that have an absolute requirement for Ca^{2+}, they may be as important as PKC and calmodulin in Ca^{2+}-mediated regulation of cellular function (Murachi, 1983). In this regard, it has been demonstrated that generation of the PKC

metabolite (PKM) by the action of calpain is essential in the exocytosis of granule contents from human neutrophils (Melloni et al., 1985; Melloni et al., 1986; Pontremoli et al., 1990a,b). Protein kinase M is Ca^{2+}/phospholipid independent and can translocate to the cytosol where it may phosphorylate soluble proteins. However, it should be noted that PKM is subject to rapid degradation by other cellular proteases and may simply be the initial step in the down-regulation of this enzyme (Kikkawa and Nishizuka, 1986).

Calpastatin is a collective name given to an endogenous and ubiquitous family of calpain inhibitors. These inhibitors are calpain-specific and are completely ineffective on trypsin, chymotrypsin and papain (reviewed by Murachi, 1983). Like calpains, calpastatin is activated by calcium, but the inhibitor does not affect the nature of the Ca^{2+}-dependence of calpain or sequester calcium making it unavailable to the protease. Instead, in the presence of calcium, calpain and calpastatin form a large aggregate that does not have proteolytic activity. Aggregate formation is reversible depending on the intracellular Ca^{2+} concentration (Murachi, 1983). Therefore, regulation of calpastatin activity may have important implications for cellular calpain and PKC activities.

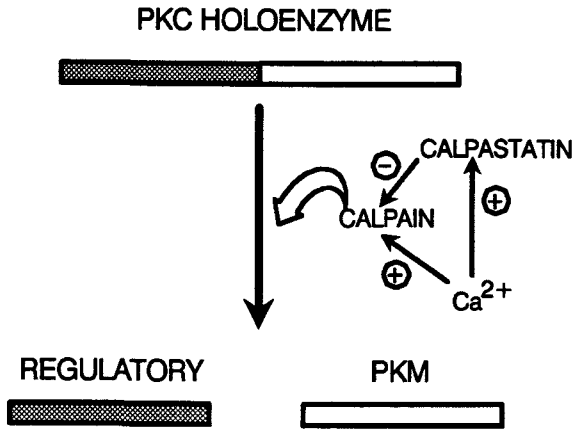

Fig. 3 Functional interrelationships between PKC, calpain and calpastatin. The calcium activated neutral protease (calpain) hydrolyzes the protein kinase C (PKC) holoenzyme, generating a regulatory subunit and a constitutively active catalytic subunit of PKC. Calpain activity is inhibited by it's endogenous inhibitor, calpastatin, which is also calcium activated.

Cellular substrates of protein kinase C

There are undoubtedly as many substrates for protein kinase C as there are cellular functions of this enzyme. This review will focus on those substrates that are involved in exocytotic processes. Robinson (1991) provides a speculative model for the involvement of three specific protein kinase C substrates in neurotransmitter release: B-50, dephosphin and the myristoylated alanine rich C kinase substrate (MARCKS). All three PKC substrates are localized in nerve terminals and appear to be phosphorylated during exocytotic stimulus. Robinson (1991) proposed that B-50, MARCKS and the phosphatase, calcineurin (CN), are involved in triggering the exocytosis of neurotransmitters. Both phospho-acceptor proteins bind calmodulin (CaM) in the unphosphorylated state and release CaM upon phosphorylation. Calcineurin is a calcium-dependent phosphatase and this dependence is partially mediated by CaM. Exocytotic stimulus, either by depolarization or phorbol ester-induced, causes the activation of PKC and the phosphorylation of B-50 and MARCKS. Upon phosphorylation, both proteins release CaM, making it available to bind and activate CN. In the resting neuron, dephosphin is phosphorylated. Once activated, calcineurin rapidly dephosphorylates dephosphin, which directly mediates the exocytotic response. Once calcium returns to pre-stimulated levels, dephosphin is rephosphorylated by PKC to prime the nerve terminus for the next exocytotic stimulus (Robinson et al., 1987). This model is only speculative because there is no direct evidence implicating CN or MARCKS in neurotransmitter release. There is substantially more evidence for the direct involvement of calmodulin, B-50 and dephosphin in neurotransmitter release.

Evidence for the involvement of the MARCKS protein in the exocytosis of neurotransmitters is only correlative. Phosphorylation of this protein is stimulated upon depolarization or phorbol ester-induced stimulation of intact synaptosomes (Dunkley et

al., 1986; Dunkley and Robinson, 1986) or brain slices (Rodnight and Leal, 1990). Both of these treatments are also known to cause the release of neurotransmitters (Robinson, 1991).

Phosphorylation of B-50 has recently been correlated with neurotransmitter release from synaptosomes (Dekker et al., 1990) and hippocampal slices (Dekker et al., 1989b). Phorbol esters enhance B-50 phosphorylation and noradrenaline release, however, both activities are inhibited upon treatment with polymyxin B. In addition, when antibodies against B-50 are introduced into permeabilized synaptosomes, the release of noradrenaline is inhibited (Dekker et al., 1989a; Dekker et al., 1991).

Evidence for the involvement of CaM in triggering exocytosis is sparse and the best direct evidence is from nonneuronal models. In this regard, microinjection of anti-CaM antibodies into chromaffin cells inhibited the stimulated release of catecholamines (Kenigsberg and Trifaro, 1985). However, CaM inhibitors do not block calcium-evoked catecholamine release from PC12 cells, providing one argument against a role for this protein in exocytosis (Matthies et al., 1988).

Potential substrates of PKC that may be involved in exocytotic events in other tissues have not been evaluated. It is conceivable that one or more of the proteins involved in neurotransmitter release also participate in exocytosis in peripheral tissues. In this regard, the possible involvement of MARCKS in the $PGF_{2\alpha}$-induced secretion of OT will be evaluated.

Myristoylated Alanine Rich C Kinase Substrate (MARCKS)

The MARCKS protein is a good candidate for the PKC substrate involved in $PGF_{2\alpha}$-induced secretion of oxytocin. This protein is a specific substrate for protein kinase C only (Blackshear et al., 1987; Graff et al., 1991). It has been demonstrated that $PGF_{2\alpha}$ induces the phosphorylation of MARCKS in cultured MC-3T3-E1 osteoblasts

(Quarles et al., 1993). Additionally, this protein cross-links actin filaments (Hartwig et al., 1992), which have been shown to be involved in the exocytosis of granules from pituitary cells (Ostlund et al., 1977; Kondo, 1987; Senda et al., 1989; Van de Moortele et al., 1991). MARCKS is expressed in a wide variety of tissues, the primary sequence of the protein is known and the cDNA has been cloned in several species, including the bovine (Stumpo et al., 1989), chicken (Graff et al., 1989b), mouse (Seykora et al., 1991; Brooks et al., 1991), rat (Erusalimsky et al., 1991) and human (Harlan et al., 1991).

The bovine MARCKS protein is approximately 31 kD in size but migrates with an anomalously high M_r of 87 kD on SDS-gels (Blackshear, 1993). This is apparently due to the rodlike shape of the protein (Hartwig et al., 1992). Sequence comparisons between species indicate that there are three major regions of conservation in the MARCKS protein. The first conserved region is at the amino-terminus. This region contains the consensus sequence for amino terminal myristoylation (Towler et al., 1988) and serves to localize the protein to the membrane. The second conserved sequence, termed the MH2 domain surrounds the site of intron splicing. Differential use of this splice site accounts for the two different forms of mRNA for bovine MARCKS found on Northern blot analysis (Stumpo et al., 1989; Harlan et al., 1991). The third conserved domain consists of a 25 amino acid amphipathic alpha-helix that contains the sites for PKC phosphorylation, actin and calmodulin binding (Fig. 4; reviewed by Aderem, 1992).

Myristoylation of MARCKS

Whether the MARCKS protein is myristoylated post-translationally (Aderem et al., 1988), co-translationally (James and Olson, 1989) or both has not been resolved. Nevertheless, myristoylation of the protein is essential for its ability to associate with the plasma membrane (George and Blackshear, 1992). However, membrane association is not required for phosphorylation by protein kinase C (Graff et al., 1989a).

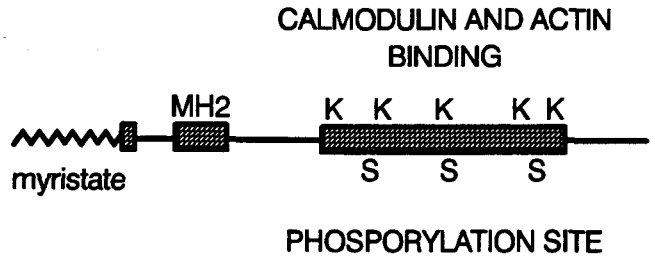

Fig. 4 The myristoylated alanine rich C kinase substrate (MARCKS). Three conserved domains are shown, including the consensus myristoylation site, the MH2 domain and the amphipathic alpha helix that binds calmodulin and actin and is the site of phosphorylation by protein kinase C. Four lysines (K) are shown on the hydrophilic side of the helix. Three phenylalanines are out of the plane of the page and make up the hydrophobic side of the helix. The helix also contains four serine residues (S), three of which are phosphorylated by PKC.

Phosphorylation of the MARCKS protein

MARCKS is a direct substrate for PKC phosphorylation both *in vitro* and *in vivo* (Blackshear et al., 1987; Graff et al., 1991). Tryptic digestion studies on the phosphorylated protein revealed four distinct phospho-serine residues that are clustered within the 25 amino acid alpha-helix (Graff et al., 1989c). A study of the PKC-catalyzed phosphorylation of a peptide analogous to the phosphorylation domain revealed that only the first, second and fourth serines are phosphorylated; the third serine is apparently in a poor context for PKC phosphorylation (Amess et al., 1992). The synthetic peptide is a substrate for PKCε as well as the conventional PKCs (Blackshear, 1993), although nothing is known about its behavior as a substrate for the other PKC isozymes.

Calmodulin binding by MARCKS

The MARCKS protein binds calmodulin in a Ca^{2+}-dependent manner and the CaM binding domain is the same as the phosphorylation domain (Graff et al., 1989c). Phosphorylation of the protein prevents calmodulin binding (Graff et al., 1989c) suggesting dephospho-MARCKS may serve as a reservoir for calmodulin, perhaps releasing CaM from membranes upon PKC activation. This concept is supported by studies with cultured neuronal cells in which activation of PKC resulted in increased cytosolic immunoreactive calmodulin with a concomitant decrease in membrane-associated calmodulin (Mangels and Gnegy, 1990). These results also support the model for neurotransmitter release proposed by Robinson (1991). MARCKS may also serve to concentrate CaM in specific cellular locations (e.g. nerve terminals).

Implications of actin binding by MARCKS

Hartwig et al. (1992) demonstrated that dephosphorylated MARCKS could bind and cross-link filamentous, but not globular actin *in vitro*. The phosphorylated form of the protein bound actin weakly, but did not cross-link filaments. In nonmuscle cells, actin exists in a dynamic equilibrium between filamentous and monomeric form, with about 50% in each form (Bray et al., 1986). A dense, subcortical network of actin filaments exists in several secretory cell types and disruption of this network occurs under exocytotic stimulus (Schlessinger and Geiger, 1981; Tramontano et al., 1982; Sobue et al., 1988). Orci et al. (1972) demonstrated that when the submembranous actin filament-rich "cell web" in pancreatic β cells is disrupted by cytochalasin B, glucose-stimulated insulin secretion increases. These results suggest that the subcortical actin filaments may obstruct the movement of secretory granules to the plasma membrane and disruption of this network may be required to allow exocytosis of granular contents.

STATEMENT OF THE PROBLEM

Of all mammalian embryos, 25-55% are lost early in gestation and much of this loss appears to be due to inadequate function of the corpus luteum (Niswender and Nett, 1988). Efforts to understand the regulation of the life span of this transient endocrine gland has important implications for improving reproductive efficiency in domestic animals.

Regression of the corpus luteum in ruminants is a precisely regulated process that involves a positive feedback between uterine $PGF_{2\alpha}$ and luteal oxytocin as initially described by McCracken (1984). It has been demonstrated both *in vivo* and *in vitro* that $PGF_{2\alpha}$ causes a release of OT from the ruminant corpus luteum, however, the mechanism by which $PGF_{2\alpha}$ induces the release of this peptide is not well understood. Preliminary experiments in our laboratory implicate protein kinase C as a second messenger involved in the $PGF_{2\alpha}$-induced secretion of oxytocin from the bovine corpus luteum (Cosola-Smith et al., 1990).

Protein kinase C exists as a family of multiple subspecies having closely related structures. At least ten isoforms of this enzyme have been described (Nishizuka, 1992), but it is not known which, if any, PKC isozymes are present in the bovine corpus luteum. Elucidation of the PKC isozymes present in the corpus luteum will provide some insight into the possible function of this enzyme in the corpus luteum. In addition it will be necessary to clearly establish a correlation between activation of PKC and the $PGF_{2\alpha}$-stimulated secretion of OT from the bovine corpus luteum.

Calpains and their endogenous inhibitor calpastatin may play an important role in the regulation of PKC activity. Therefore it is important to determine whether treatment with $PGF_{2\alpha}$ causes a change in the level of these proteins present in the corpus luteum.

If protein kinase C is involved in the secretion of OT from the bovine corpus luteum, it will be necessary to determine the cellular substrates phosphorylated by this

enzyme. The MARCKS protein offers itself as a good potential substrate for PKC phosphorylation in this tissue because it is a ubiquitously distributed, specific substrate for PKC and has been found in other bovine tissues. Experiments will be conducted to determine the presence and distribution of the MARCKS protein the bovine CL and whether the phosphorylation status of this protein is affected by treatment with $PGF_{2\alpha}$ and/or the PKC activating phorbol ester.

Evidence for the $PGF_{2\alpha}$-induced secretion of OT from the bovine corpus luteum is abundant. However, the subcellular events that regulate OT secretion are not well understood. The data presented herein provides the groundwork for future research related to the regulation of OT secretion from the bovine corpus luteum, and may prove useful in the study of exocytosis of peptide or protein hormones from a variety of endocrine tissues.

IMMUNOCHEMICAL CHARACTERIZATION AND CELLULAR DISTRIBUTION OF PROTEIN KINASE C ISOZYMES IN THE BOVINE CORPUS LUTEUM

Abstract

Research was conducted to determine the nature of the isoforms of protein kinase C (PKC) present in the bovine (*Bos taurus*) corpus luteum (CL) and their subcellular distribution. Western blot analysis was performed using isozyme-specific polyclonal antibodies and revealed that the bovine corpus luteum contains the α and ε isozymes of PKC, but not the β, γ, δ or ζ isozymes. Subcellular fractionation demonstrated that the α and ε isozymes of PKC were present in both the cytosolic and plasma membrane fractions. Densitometric measurements of Western blots of the subcellular luteal PKC fractions indicated that PKCα was found predominantly in the cytosolic fraction, whereas, the majority of PKCε was associated with the plasma membrane.

Introduction

Molecular cloning has revealed that protein kinase C (PKC) exists as a family of multiple subspecies having closely related structures. At least ten isoforms of this enzyme (α, βI, βII, γ, δ, ε, η, λ, θ and ζ) have been identified (Nishizuka, 1992). These isozymes exhibit slightly different modes of activation, kinetic properties and tissue distribution. The α, βI, βII, and γ isozymes have been designated conventional PKCs and are activated by calcium, phospholipids and diacylglycerol (or phorbol ester). The δ, ε, η and θ isozymes, designated novel PKCs, lack the calcium-binding domain found in the cPKCs, however, they are activated by phospholipids and diacylglycerol or phorbol ester. The λ and ζ isozymes are further atypical in that they are not affected by calcium, diacylglycerol or phorbol ester, but are phosphatidylserine dependent.

Protein kinase C has been shown to be involved in progesterone production (Wiltbank et al., 1989) as well as oxytocin secretion (Cosola-Smith et al., 1990) by the corpus luteum. However, the PKC isozymes actually present in the bovine (*Bos taurus*) corpus luteum have not been elucidated. Also, the distribution of PKC isozymes within bovine luteal cells is unknown. Thus, the purpose of the present research was to determine which PKC isoforms are present and their subcellular distribution within the bovine corpus luteum.

Materials and Methods

Beef heifers (12-16 months of age) were checked twice daily for behavioral estrus with a vasectomized bull. Normally cycling heifers were restrained on day 8 of the cycle (day of detected estrus = day 0) and 3 ml of 2% lidocaine were injected into the coccygeal spinal cord to induce caudal epidural anesthesia. Corpora lutea (CL) were removed per vaginum as modified from the procedure described by Casida (1959), dissected free of connective tissue, frozen in liquid nitrogen and stored at -80°C until analyses were performed.

For the isolation of PKC, luteal samples (0.5 g) were homogenized in 4 ml extraction buffer (4°C) containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol (β-ME), 0.5% Triton X-100, 25 μg/ml leupeptin and 25 μg/ml aprotinin. After the homogenate was incubated on ice for 30 min, it was centrifuged at 20,000 x *g* for 20 min at 4°C. Supernatants (containing PKC) were filtered through glass wool and stored at 4°C until partial purification was performed later the same day.

Subcellular fractionation of luteal tissue was accomplished by differential and discontinuous sucrose gradient centrifugation as described by Gospodarowicz (1973) and Bramley and Ryan (1978). As demonstrated by Gospodarowicz and confirmed in our

laboratory (Zelinski et al., 1988), luteal subcellular fractions separated by this technique are relatively free of contamination by other fractions. Supernatants were stored at 4°C until partial purification was performed later the same day.

Supernatants from whole CL and subcellular fractions were partially purified on 0.5 g DEAE cellulose columns which had been prewashed with 2 ml wash buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA and 10 mM β-ME. After addition of sample, columns were washed with 5 ml wash buffer and eluted with 4 ml elution buffer (wash buffer containing 0.2 M NaCl). Samples were stored at -20°C until Western blot analysis was performed.

Western blot analysis was performed on partially purified luteal samples to characterize PKCs endogenous to the CL. Rat and bovine brain tissues were included as positive controls. Samples were analyzed for protein content as described by Bradford (1976) using bovine serum albumin as the standard. Partially purified samples (100 μg protein) were separated by 12% SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970). Proteins were transferred onto a nitrocellulose membrane (0.45 μm, Amersham, Arlington Heights, IL) according to the methods of Towbin et al. (1979) in transfer buffer containing 20 % methanol, 25 mM Tris-HCl and 192 mM glycine. After transfer, blots were washed with Tris-buffered saline (TBS) containing 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl and incubated at 25°C for 1.5 h with blocking solution (1% BSA-TBS). Membranes were then incubated with isozyme-specific antibodies [rabbit anti-peptide PKC antibodies (1:250 dilution in TBS containing 0.5% Tween-20), affinity purified, Gibco BRL, Grand Island, NY] for 2 h at 25°C, followed by incubation with alkaline phosphatase-conjugated mouse anti-rabbit immunoglobin (1:3000 dilution, Bio-Rad, Richmond, CA) for 1 h at 25°C. Blots were washed four times with TBS containing 0.5% Tween-20 and 3X with TBS. Peroxidase activity was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (alkaline phosphatase conjugate substrate kit; Bio-Rad). Peptides against which the antibodies were prepared (Gibco

BRL) were used as negative controls as a specific blocker of antibody binding. Levels of protein kinase C in the subcellular fractions were quantified by densitometric scanning of the Western blot using the model 1650 transmittance/reflectance scanning densitometer (Bio-Rad) and the Hoefer GS 350 Data System (Hoefer Scientific Instruments, San Francisco, CA).

Results

Luteal samples were examined for the presence of PKC isozymes α, β, γ, δ, ε and ζ. Western blot analysis revealed that the bovine corpus luteum expressed α and ε isozymes of PKC (Fig. 5, lanes A), but did not contain β, γ, δ or ζ. As expected, PKC specific bands were observed for all isozymes in the rat brain samples (Fig. 5, lanes B), although the signal for ζ was very weak. Similarly, specific PKC bands were found for all isozymes in the bovine brain controls (data not shown). In addition, molecular weights of the PKC-specific bands were consistent with those previously reported (Kikkawa et al., 1989). Preincubation of isozyme-specific antibodies with the peptides against which they were prepared caused the disappearance of the PKC specific band in Western blots, indicating the antibody specificity (data not shown).

Because the first Western blot indicated the presence of PKC α and ε in the bovine CL, a second experiment was performed to determine the distribution of these isozymes within luteal cells. The α and ε isozymes were present in both the cytosolic and plasma membrane fractions of the bovine CL (Fig. 6). The signal for PKCα was stronger in the cytosol (867 densitometric units) than in the membrane fraction (393 densitometric units). Conversely, the signal for PKCε was stronger in the plasma membrane fraction (3257 densitometric units) than in the cytosolic fraction (1471 densitometric units, Fig. 6C and D). Densitometric values for PKC α could not be compared to those for PKCε in each fraction because binding characteristics of the primary antibodies were different.

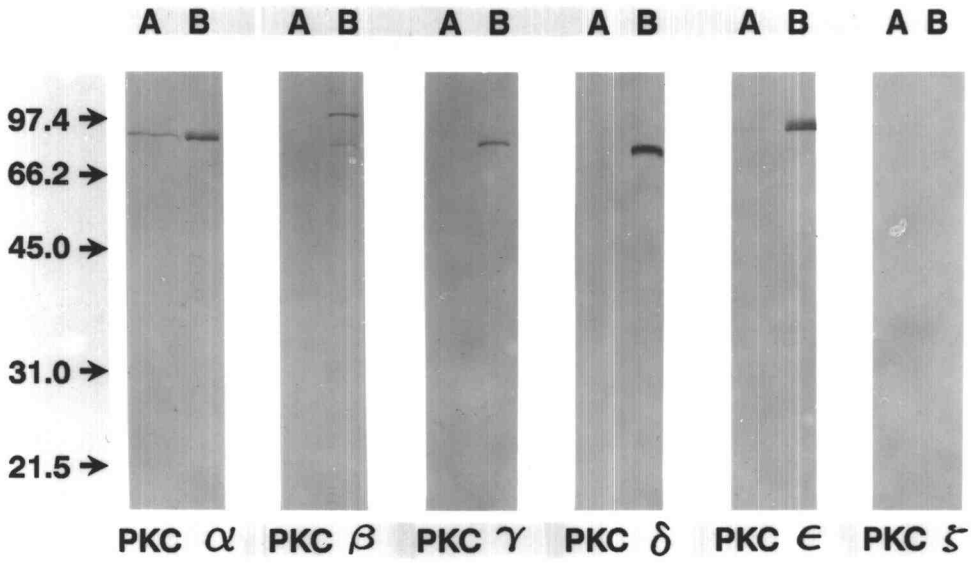

Fig. 5 Western blot of bovine corpus luteum (lanes A) and rat brain (lanes B) samples using isozyme-specific PKC antibodies.

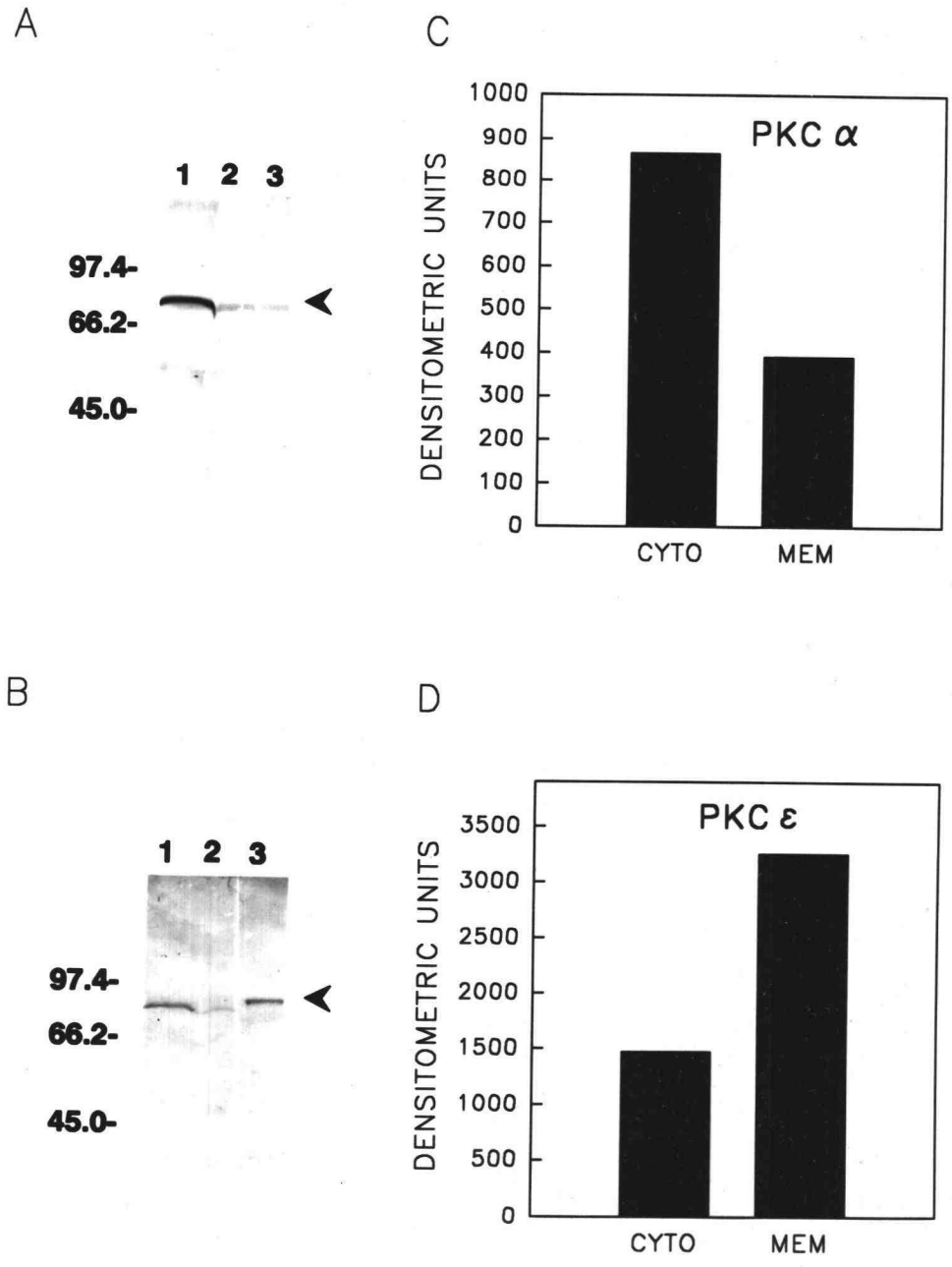

Fig. 6 Western blots of PKCα (A) and PKCε (B) in subcellular fractions of the bovine corpus luteum; lane 1: whole CL, lane 2: cytosol, lane 3: membrane. Densitometric analysis for Western blots in A and B are depicted in C and D, respectively.

Discussion

The unique PKC content and distribution in the bovine CL has not been previously reported. Our data indicate that the CL contains the α and ε isozymes of PKC. Upon cell stimulation PKC translocates to the membrane (Kraft and Anderson, 1983) where it can be activated by diacylglycerol and phospholipids and subsequently affect distal functions within the cell. Because PKC has been shown to play a role in the exocytosis of oxytocin from the bovine CL, it was of interest to examine the subcellular distribution of PKC isozymes. More PKCα was found in the cytosol than the membrane. In contrast, PKCε was associated more with the plasma membrane than the cytosolic fraction.

While the roles of PKCα and ε in the bovine CL are not known, both isozymes have been implicated in exocytotic events in other tissues. Binding of gonadotropin-releasing hormone to its receptor is known to stimulate phosphoinositide turnover, mobilize calcium and activate PKC to cause gonadotropin release from the pituitary (Naor, 1990). Recently it has been shown that activation of PKCα markedly and consistently stimulates luteinizing hormone release from permeabilized pituitary cells *in vitro* (Naor et al., 1989). The authors concluded that PKCα may be "overexpressed" in the pituitary because one of the main functions of this gland is the exocytosis of peptide or protein hormones. A similar system involving PKCα may be acting in the bovine CL because prostaglandin $F_{2\alpha}$ has been shown to cause release of the neuropeptide oxytocin from this tissue *in vivo* (Schallenberger et al., 1984) and *in vitro* (Abdelgadir et al., 1987). Like gonadotropin-releasing hormone, prostaglandin $F_{2\alpha}$ binds to its receptor and stimulates phosphoinositide turnover (Leung et al., 1986; Davis et al., 1987), causes calcium influx from intracellular and extracellular stores and activates PKC (Goins and Jimenez de Asua, 1991). Electron microscopy has verified that prostaglandin $F_{2\alpha}$ causes exocytosis of oxytocin-laden secretory granules from bovine large luteal cells (Chegini and Rao, 1987). In addition, previous work in our laboratory has demonstrated that increased

calcium and activation of PKC are involved in this oxytocin release (Cosola-Smith et al., 1990). Therefore, it is possible that the highly expressed PKCα in the bovine CL functions in part to regulate the secretion of the neuropeptide, oxytocin.

Messenger RNA for PKCε is expressed at high levels in the brain, lung and heart of the rat and at low levels in various other tissues (Schaap et al., 1989). To our knowledge, this is the first report of the presence of this isozyme in the corpus luteum. PKCε is activated in the same phospholipid-, diacylglycerol (or phorbol ester)-dependent manner as the conventional PKCs but is not calcium dependent (Konno et al., 1989). This calcium-independent isozyme is also subjected to different regulation in response to phorbol esters and thyrotropin releasing hormone than the conventional PKCs in rat pituitary tumor GH_4C_1 cells, which have been shown to contain the α, β and ε isozymes of PKC (Akita et al., 1990). All three PKC isozymes present in GH_4C_1 cells mediate the secretion of prolactin and growth hormone in response to phorbol esters or thyrotropin releasing hormone, and these investigators have shown that secretion of prolactin is significantly maintained even in calcium-depleted cells. This secretion of prolactin is inhibited by H7 and staurosporine (PKC inhibitors), suggesting that exocytosis of this peptide hormone by GH_4C_1 cells is a PKC-dependent process which is at least partly calcium-independent, thus implicating PKCε. In addition to its unique mode of activation and regulation, PKCε differs from conventional PKCs in substrate specificity (Pears et al., 1991).

While both PKCα and ε have been shown to be involved in hormone dependent signal transduction, these isozymes are subjected to different modes of activation, regulation and substrate specificity allowing for diverse cellular responses. Wiltbank et al. (1989) have shown that specific activation of PKC caused an acute and dose dependent inhibition of progesterone production in luteinizing hormone-stimulated ovine small luteal cells and unstimulated large cells. There is some indication that PKC also acts as a mediator in prostaglandin $F_{2\alpha}$-induced secretion of oxytocin from bovine luteal cells

(Abdelgadir et al., 1987). It remains to be determined which PKC isozymes are involved in these events in the bovine corpus luteum.

This research has elucidated the PKC isozymes present in the bovine CL and their unstimulated cellular distribution. However, because the CL is composed of both small and large luteal cells, it remains to be determined which cell types in the bovine CL contain these PKC isozymes.

INVOLVEMENT OF PROTEIN KINASE C, CALPAINS, AND CALPASTATIN IN PROSTAGLANDIN $F_{2\alpha}$-INDUCED OXYTOCIN SECRETION FROM THE BOVINE CORPUS LUTEUM

Abstract

An experiment was conducted to determine whether prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced secretion of oxytocin (OT) by the bovine corpus luteum (CL) was associated with changes in the activities of protein kinase C (PKC), calpains and calpastatin. On day 8 of the estrous cycle (estrus = day 0), beef heifers were restrained and given a 500 mg i.v. injection of cloprostenol, a $PGF_{2\alpha}$ analog. Corpora lutea were surgically removed from beef heifers at 0, 2, 7.5 or 30 min (n = 4 animals per time period) after cloprostenol injection. Blood samples were collected before injection and at frequent intervals after injection. Distribution of PKC activity in cytosol and membrane fractions and activities of μ-calpain, m-calpain and calpastatin were determined for all CL. Oxytocin was measured in plasma and tissue by radioimmunoassay.

Relative to mean plasma levels of OT at time 0 (85±7 pg/ml), peak plasma levels occurred between 1.5 and 10 min (270±36 pg/ml) for all animals. Mean luteal concentration of OT (ng/g) was greater at 0, 2 and 7.5 min (145±27, 232±82 and 269±115, respectively), than at 30 min (93±33), but differences in tissue OT over time were not significant ($p>0.05$). Protein kinase C activities (percentage over non-activated controls) in the membrane or cytosolic fractions did not differ significantly among the times of CL removal; however, membrane PKC activity was positively correlated with plasma level of OT at time of CL removal ($r = 0.82$, $p<0.0025$). Luteal m-calpain activity was approximately twice that of μ-calpain at each time point ($p<.001$), although activities of the individual calpains over time after $PGF_{2\alpha}$ injection did not change. Calpastatin activity was significantly higher at 30 min (515±28 units/g tissue) than at 0, 2, or 7.5 min (373±26, 423±26 and 426±24 units/g tissue, respectively). Activity of PKC in the

membrane appears to be positively correlated with OT secretion from the bovine CL and increased calpastatin activity after $PGF_{2\alpha}$ injection may inhibit calpains present in the CL, thereby maintaining an active pool of PKC.

Introduction

Prostaglandin $F_{2\alpha}$ stimulates secretion of oxytocin (OT) from the ruminant corpus luteum (CL) *in vivo* (Schallenberger et al., 1984; Walters et al., 1984; Lamsa et al., 1989; Flint et al., 1990) and *in vitro* (Abdelgadir et al., 1987; Chegini and Rao, 1987). It has been demonstrated that the action of $PGF_{2\alpha}$ on the CL provokes phosphoinositide turnover with resultant increases in diacylglycerol (DAG) and intracellular Ca^{2+} concentrations (Leung et al., 1986; Davis et al., 1987). Because DAG and Ca^{2+} activate protein kinase C (PKC), it is hypothesized that this enzyme may regulate the secretion of OT from large luteal cells, which are the source of this nonapeptide (Guldenaar et al., 1984). This premise is supported in part by the data of Cosola-Smith et al. (1990), who found that *in vitro* treatment of bovine luteal tissue with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, which directly activates PKC, mimicked the effects of $PGF_{2\alpha}$ by inducing the secretion of OT. In addition, PKC has been shown to play a role in secretion and exocytosis events in a wide variety of cell types, including several endocrine tissues (Kikkawa and Nishizuka, 1986; Naor et al., 1989).

Calpains, ubiquitous calcium-activated neutral proteases, hydrolyze membrane associated PKC molecules to lower molecular weight forms of the enzymes (PKMs) that are constitutively active (Melloni et al., 1986; Nishizuka, 1986). The susceptibility of PKC to hydrolysis by calpain increases significantly under conditions in which PKC is active (Suzuki, 1991). These proteases may serve as an additional point of regulation of PKC activity. However, the significance of this proteolytic activation is unknown, and may simply be the first step in the down-regulation of PKC (Nishizuka, 1988). Micro (μ)-

and milli (m)-calpains, activated by micro- and millimolar calcium concentrations, respectively, are inhibited by their endogenous inhibitor, calpastatin, which is also calcium dependent (Murachi, 1983).

Based upon existing data delineated above, we propose a model (Fig. 7) which integrates the functional relationships between PKC, calpains and calpastatin in $PGF_{2\alpha}$-stimulated bovine luteal cells. According to this model, $PGF_{2\alpha}$ binds to its receptor on the plasma membrane of the large luteal cell and acts through a G-protein to activate phospholipase C, which in turn converts phosphatidylinositol-4,5-bisphosphate to diacyl-glycerol (DAG) and inositol trisphosphate. Inositol trisphosphate mobilizes calcium from intracellular stores in the endoplasmic reticulum, which is involved in the activation of PKC, μ- and m-calpains and calpastatin. Membrane bound PKC is activated by calcium, DAG and phosphatidylserine and may be involved in the exocytosis of oxytocin from large luteal cells. Alternatively, activated PKC may be hydrolyzed by calpains to its lower molecular weight form (PKM), which is intrinsically active and may be the biologically important form of the enzyme that promotes exocytosis. The objective of the present study was to examine temporal changes in bovine luteal PKC, calpain and calpastatin activities associated with $PGF_{2\alpha}$-induced secretion of oxytocin.

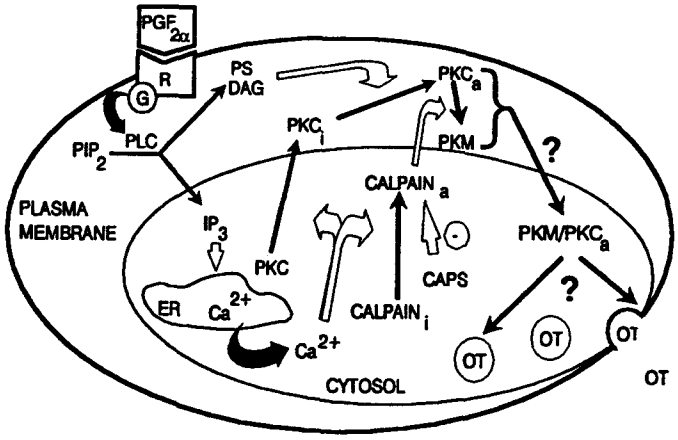

Fig. 7 Proposed model demonstrating the mechanism of action by which $PGF_{2\alpha}$ stimulates the release of oxytocin (OT) from large luteal cells in cattle. Model includes possible functional relationships between PKC, calpains and calpastatin (CAPS) in this exocytotic event. Abbrevs: Receptor (R), G-protein (G), Phospholipase C (PLC), Phosphatidylinositol-4,5-bisphosphate (PIP_2), Diacylglycerol (DAG), Inositol trisphosphate (IP_3), Endoplasmic reticulum (ER), Inactive protein kinase C (PKC_i), Active PKC (PKC_a), Inactive calpain ($CANP_i$), Active calpain ($CANP_a$), Phosphatidylserine (PS) and constitutively active PKC metabolite (PKM).

Materials and Methods

Animals

Sixteen beef heifers (12-16 months of age) were checked twice daily for estrus using a vasectomized bull. Each heifer exhibiting estrous cycles of normal duration was restrained on day 8 of the cycle (day of detected estrus = day 0) and the jugular vein catheterized using a 16 gauge 8.3 cm Angiocath catheter (Becton Dickinson Deseret Medical, Sandy, UT). The catheter was kept open by infusion of a solution containing 3.5% sodium citrate/0.2% oxytetracycline. A jugular blood sample was collected immediately after catheterization and designated as "background"; all blood samples were collected in 10 ml heparinized tubes. Ethylenediamine-tetraacetic acid (EDTA; 0.5 M, 20 ml) and 1,10-phenanthroline (5 mg/ml in ethanol, 10 ml) were added immediately to all

blood samples to prevent oxytocinase activity (Kumarasen et al., 1974). Lidocaine (2%) was used to induce caudal epidural anesthesia and an intravaginal incision was made through which the corpus luteum was removed as described by Casida (1959). To preclude a possible effect of the surgical procedure on luteal OT secretion, the surgery, except for the actual enucleation of the CL, was performed before the injection of $PGF_{2\alpha}$ analog at time 0. All surgical procedures were carried out under aseptic conditions. A blood sample designated as 0 min was taken and 500 μg of cloprostenol, a $PGF_{2\alpha}$ analog (Estrumate, Mobay Corp, Shawnee, KS) were immediately injected via the catheter. Corpora lutea were removed at 0 (immediately prior to cloprostenol injection), 2, 7.5 or 30 min after cloprostenol injection (n = 4 animals per time period) and dissected free of connective tissue, weighed, frozen in liquid nitrogen and stored at -80°C. Additional blood samples were taken at 1.5, 3, 6, 10, 15, 20, 25, 30, 35 and 40 min after cloprostenol (hereafter referred to simply as $PGF_{2\alpha}$) injection. Blood samples were centrifuged at 4°C and plasma stored at -20°C.

All chemicals were purchased from Sigma (St. Louis, MO), Boehringer Mannheim (Indianapolis, IN), Bio-Rad (Richmond, CA), J.T. Baker Chemical Co. (Phillipsberg, NJ) or Gibco BRL (Grand Island, NY) unless otherwise noted. Radiochemicals were purchased from New England Nuclear (Boston, MA).

Oxytocin radioimmunoassay

Oxytocin was measured in plasma and tissue samples by radioimmunoassay as described by Abdelgadir et al. (1987) using OT antibody generously provided by Dr. Dieter Schams, Technical University of Munich, Freising-Weihenstephan, Germany. Oxytocin was extracted from plasma by the method of Schams (1983) and from CL tissue according to Tsang et al. (1990). Mean extraction efficiency for plasma was 67.6% and for tissue 72.3% as determined by the addition of [^{3}H]-OT. Oxytocin values determined

by RIA were corrected for losses due to extraction. Plasma sample volumes were 10-25 µl per tube and tissue sample volumes were 100 µl per tube; all samples were brought to a final volume of 200 µl with assay buffer (0.05 M sodium phosphate, 50 mM EDTA and 0.5 mg/ml gelatin). Intra- and interassay coefficients of variation, determined from aliquots of a serum pool containing a concentration of OT that was near the midpoint of the standard curve, were 8.4 and 6.4%, respectively. The sensitivity of the assay, defined as the lowest concentration of ligand that could be distinguished from a sample containing no ligand, was 0.25 pg/tube.

Subcellular fractionation of the bovine corpus luteum

Luteal samples (0.4 g) were homogenized (4°C) in 4 ml buffer A [20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol (β-ME), 25 µg/ml leupeptin and 25 µg/ml aprotinin] in a Dounce homogenizer. Differential centrifugation was performed to separate the cytosol and membrane fractions. The homogenate was centrifuged at 1000 x *g* for 10 min, the supernatant was removed and the pellet (nuclear fraction) was discarded. The resultant supernatant was centrifuged at 100,000 x *g* for 1 h and the new supernate (cytosolic fraction) was filtered through glass wool. The pellet from this centrifugation was resuspended in 2 ml buffer A containing 0.5% Triton X-100 and incubated on ice for 1 h to free membrane-bound proteins. This membrane fraction was then centrifuged at 100,000 x *g* and the supernatant containing membranous proteins was filtered through glass wool. All supernatants were stored at 4°C until partial purification of PKC was performed later the same day.

Partial purification of protein kinase C from the bovine corpus luteum

Supernatants from subcellular fractionation were added to 0.5 g DEAE cellulose columns which had been prewashed with 2 ml wash buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA and 10 mM β-ME. After addition of sample, columns were washed with 5 ml wash buffer, then eluted with 4 ml elution buffer (wash buffer containing 0.2 M NaCl). Samples were stored at 4°C overnight and then assayed in triplicate for PKC activity.

Partial purification of calpains and isolation of calpastatin

Corpus luteum samples (1.5 g) were homogenized (4°C) in 10 ml buffer C [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM β-ME, and 150 nM pepstatin A] with a Tekmar Tissumizer in four 10 sec bursts. The homogenate was centrifuged at 1620 x *g* for 30 min at 4°C. After centrifugation, 1 ml of supernatant was removed for isolation of calpastatin and protein determination. μ- and m-Calpains were isolated by phenyl-sepharose column chromatography as described by Gopalakrishna and Barsky (1985) and modified in our laboratory (Ou and Forsberg, 1991; Ilian and Forsberg, 1992). m-Calpain was eluted with 4 ml buffer B [20 mM Tris-HCl (pH 7.5) and 1 mM EGTA] supplemented with 0.1 M NaCl. The column was washed with 2 ml buffer B containing 0.1 M NaCl. μ-calpain was then eluted with 4 ml buffer B without NaCl. The above procedures were performed at 4° C.

Calpastatin was prepared from 500 ml supernatant removed from each calpain sample before column purification. Samples were heated to 100°C for 5 min to inactivate endogenous calpains and other proteases and microcentrifuged at 10,000 x *g* for 5 min at 4°C. Supernatants were removed and assayed in duplicate for calpastatin activity.

Protein kinase C activity assay

Samples were assayed for PKC activity by measuring the transfer of ^{32}P from [γ-^{32}P] ATP to a specific substrate, histone IIIS, by modification of the mixed micelle method described by Bell et al. (1986). The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 200 µg/ml histone IIIS, 10 µM [γ-^{32}P] ATP (2.5 µCi/ml), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3% Triton X-100, 0.28 mg/ml phosphatidylserine, 0.62 mg/ml dioleoylglycerol, 5 µM microcystin and was brought to a final volume of 200 µl with double distilled water. The reaction was initiated by the addition of 50 µl of the partially purified PKC sample (each sample was assayed in triplicate). Samples in the reaction mixture were incubated at 27°C for 10 min (this incubation time was found to be in the middle of the linear part of the time course curve for phosphorylation of histone) and the reaction stopped by the addition of 1 ml cold BSA (1 mg/ml) and 1 ml cold 25% trichloroacetic acid (TCA). Tubes were incubated on ice for 1 h to allow the precipitation of proteins and subsequently vacuum filtered over glass fiber filters. The filters were washed with 10 ml 10% TCA and counted in a Beckman LS 6000 liquid scintillation counter to determine phosphorylation of histone by ^{32}P. Each assay contained controls in which the specific PKC activators, $CaCl_2$, diacylglycerol and phosphatidylserine were not added. Removal of these specific PKC activators caused a 47% reduction in cytosolic and a 82% reduction in membrane associated kinase activity compared to activated samples, with the remaining activity probably due to the presence of other kinases (data not shown). The validity of the assay was further demonstrated by the addition of the PKC inhibitor peptide (1 µM PKC 19-36, Gibco BRL) and staurosporine (1 µM), which caused a 37 and 62% reduction in kinase activity, respectively.

Calpain and calpastatin activity assays

μ- and m-Calpain activities were determined using methods which were initially established by Gopalakrishna and Barsky (1985) and modified in our laboratory (Ou and Forsberg, 1991; Ilian and Forsberg, 1992). One milliliter excess denatured casein substrate (8mg/ml in 20 mM Tris-HCl, pH 7.5, and 10 mM β-ME) was added to 3 ml of the μ-calpain or m-calpain eluant (partially purified luteal sample,1.125 g equivalent) in the presence of 2.4 mM $CaCl_2$. Duplicate samples were incubated at 25°C for 30 min; 1 ml of 36% (w/v) TCA was then added to each sample. The TCA-soluble digested products were measured by the dye binding method of Bradford (1976). One unit of calpain activity was defined as the amount of enzyme that caused a calcium-dependent increase of 0.1 unit of absorbance (595 nm)/g of tissue in 30 min at 25°C. Control samples that contained 2 mM EDTA in place of the $CaCl_2$, had an absorbance of 0, indicating no proteolysis of the casein substrate.

Calpastatin activity was determined by the method of Nakamura et al. (1988). Duplicate aliquots (100 ml) of the prepared calpastatin samples were added to 400 ml of partially purified bovine CL m-calpain containing 2.4 mM $CaCl_2$ and incubated at 25°C for 5 min. Three hundred microliters of denatured casein substrate were then added and hydrolysis of casein assessed as described above for the calpain assay. One unit calpastatin activity was defined as the amount that inhibited 1 unit bovine CL m-calpain activity. Samples to which calpastatin was not added served as controls and exhibited an increase in calpain activity as indicated by enhanced proteolysis of casein.

Statistical analysis

Differences between μ- and m-calpain activities as well as differences between basal and peak plasma OT levels were determined by a paired Student's *t*-test. All other

data were analyzed statistically by one-way analysis of variance. Differences among time intervals for the characteristics analyzed were tested for significance using an F-protected least significant difference test. Relationship between plasma oxytocin and membrane PKC activity was determined by simple linear regression using the least squares method. All statistical analyses were performed by Statgraphics statistical graphics program (STSC Inc., Rockville, MD).

Results

Prostaglandin $F_{2\alpha}$-induced secretion of oxytocin by the CL was confirmed and quantitated by radioimmunoassay of plasma collected at various times after $PGF_{2\alpha}$ injection (Fig. 8). Time of peak plasma concentrations of oxytocin varied for each animal; all peaks occurred at the 1.5, 3, 6 or 10 min sampling times (relative to injection of $PGF_{2\alpha}$). Those animals in which the CL was removed at 0 min after $PGF_{2\alpha}$ injection (Fig. 8, 0 min) did not exhibit a peak release of OT that was higher than baseline ($p>0.05$). However, animals in which the CL was removed at later time points all had a peak OT release that was significantly higher than baseline ($p\leq0.05$). In addition, the area under the plasma OT curve (determined by integration of plasma levels of OT over time) was smaller for those cows in which the CL was removed at 0 min (4405 pg·min/ml) than for those in which the CL was removed at 2, 7.5 or 30 min (6910, 7614 and 6724 pg·min/ml) after $PGF_{2\alpha}$ injection (data not shown). The time-0 plasma oxytocin level (mean ± SE) for all animals in the study was 85±7 pg/ml. Mean peak plasma levels of OT for cows with CL removed at 0, 2, 7.5 and 30 min after injection of $PGF_{2\alpha}$ were 164± 33, 269± 55, 363± 101 and 285± 69 pg/ml, respectively. Luteal concentrations of OT were lower at 30 min than at 0, 2 or 7.5 min after $PGF_{2\alpha}$ injection; however, these differences were not significant ($p>0.05$, Fig. 9).

Protein kinase C activities in the cytosol and membrane fractions at 0, 2, 7.5, and 30 min after injection, reported as percentage of non-activated controls, are depicted in Fig 10. Although membrane PKC activity was highest at 7.5 min after injection, which approximately coincides with the time of peak OT secretion, it was not significantly different from that of membranes at other times analyzed ($p>0.05$). Membrane PKC activity was, however, positively correlated with plasma oxytocin levels at the time of CL removal ($r=0.82$, $p<0.0025$, Fig. 11). The level of PKC in the cytosol did not change over time; the mean was 213±21% of non-activated control for all animals (Fig. 10).

Activity of μ- and m-calpains in the CL did not differ over time ($p>0.05$, Fig 12). However, activity of m-calpain was significantly greater than that of μ-calpain at each time point ($p<0.001$). Mean μ- and m-calpain activities for all animals were 2.9±0.1 and 5.7±0.5 units/g tissue, respectively. Calpastatin activity in the CL was significantly greater at 30 min after $PGF_{2\alpha}$ injection than at 0, 2 or 7.5 min after injection ($p<0.02$, Fig. 13).

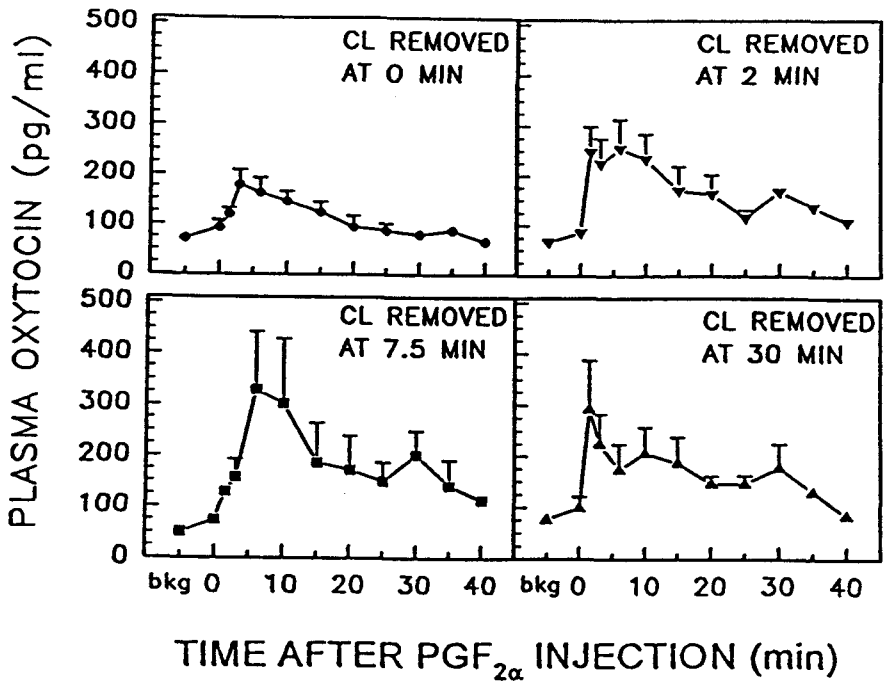

Fig. 8 Mean (± SE) plasma levels of oxytocin (pg/ml) at various times after injection of $PGF_{2\alpha}$. The time of CL removal is noted on each graph. Bkg represents background blood samples taken prior to surgery for CL removal.

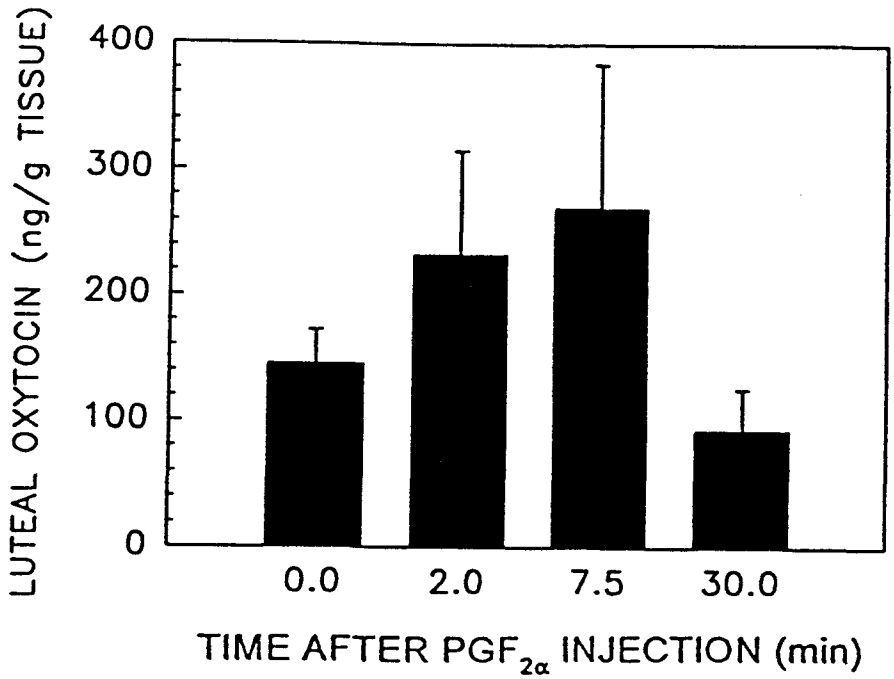

Fig. 9 Tissue levels of oxytocin (ng/g) in the bovine CL at various times after treatment with $PGF_{2\alpha}$. Each bar represents the mean ± SE of four animals.

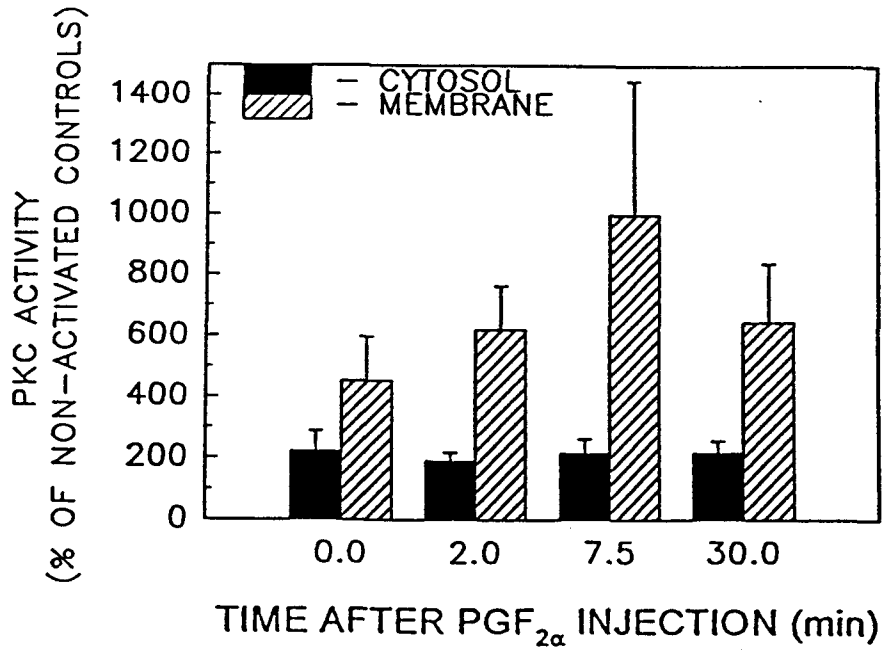

Fig. 10 PKC activity in the bovine CL expressed as a percentage of non-activated controls at various times after $PGF_{2\alpha}$ injection. Solid bars represent cytosolic PKC activity; hatched bars represent membrane PKC activity. Values are mean ± SE.

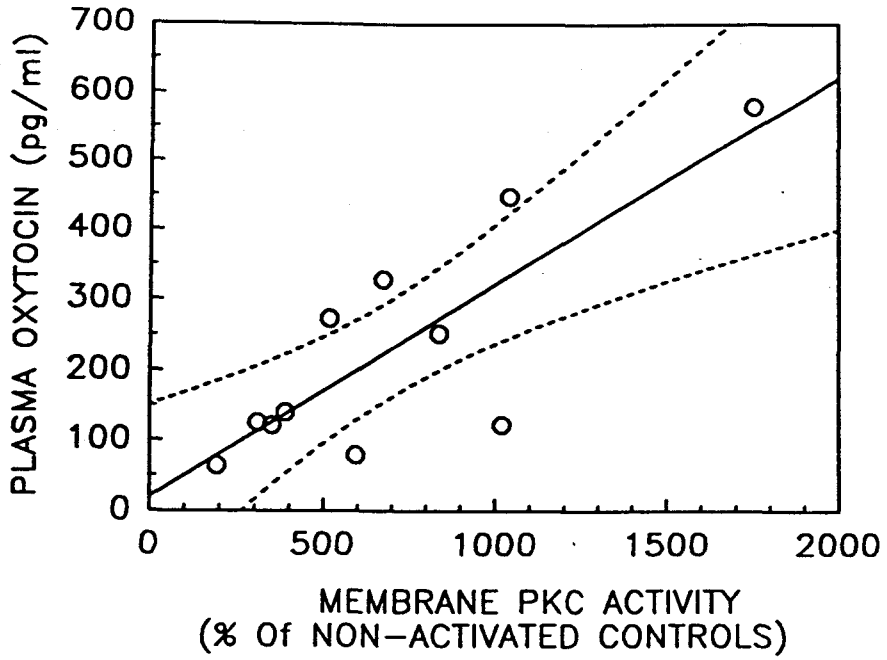

Fig. 11 Correlation between plasma oxytocin (ng/ml) and membrane PKC activity (percentage of non-activated control) at time of CL removal. The dashed line represents the 95% confidence interval. $r=0.82$, $p<0.0025$.

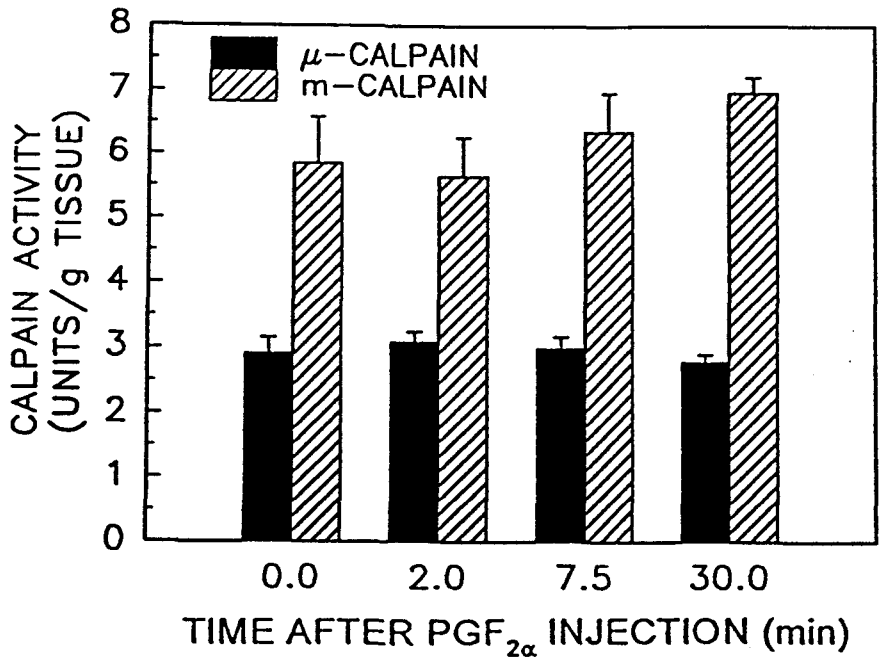

Fig. 12 Activities of μ-calpain and m-calpain (units/g tissue ± SE) in the bovine CL at various times after $PGF_{2\alpha}$ injection. One unit of calpain activity is defined as the amount of enzyme that caused a calcium dependent increase of 0.1 unit of absorbance (595 nm)/g tissue in 30 min at 25°C.

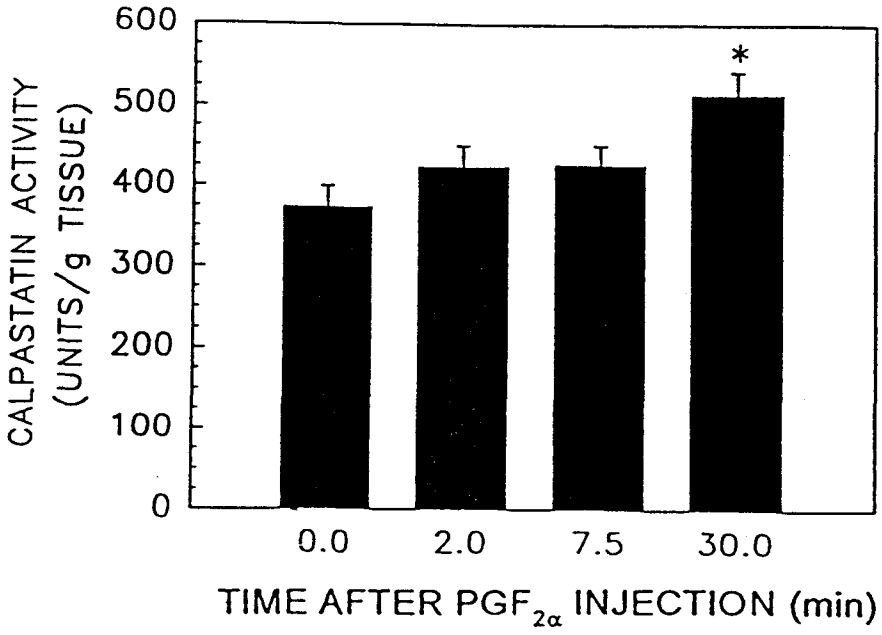

Fig. 13 Activity of calpastatin (units/g tissue ± SE) in the bovine CL at various times after $PGF_{2\alpha}$ injection. One unit of calpastatin activity is defined as the amount that inhibited 1 unit bovine CL m-calpain activity. * Denotes difference from other means ($p<0.02$).

Discussion

Hormonal regulation of PKC activity in luteal cells has not been fully elucidated; however, this enzyme appears to be activated by $PGF_{2\alpha}$ (Wiltbank et al., 1991). Previous work in our laboratory demonstrated that $PGF_{2\alpha}$ and the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, a specific activator of PKC, are both effective in stimulating *in vitro* release of OT from mid-luteal phase bovine corpora lutea (Abdelgadir et al., 1987; Cosola-Smith et al., 1990). Collectively, these data implicate a role for PKC in the $PGF_{2\alpha}$- induced release of OT from bovine luteal cells. The present study focused on the relationships between $PGF_{2\alpha}$-induced OT secretion and PKC, calpain and calpastatin activities in the bovine CL *in vivo*.

Analysis of plasma concentrations of oxytocin at various times after $PGF_{2\alpha}$ injection confirmed that $PGF_{2\alpha}$ did indeed cause a maximal release of oxytocin between 1.5 and 10 min after injection. Those animals in which the CL was removed at 0 min relative to injection of $PGF_{2\alpha}$ exhibited a smaller and narrower peak of oxytocin than those in which the CL was removed at later times, suggesting that most of the oxytocin release observed was from the ovary and not of neurohypophyseal origin. This is supported by the observation of Schams et al. (1985), who reported that the posterior pituitary released only minute quantities of OT after prostaglandin injection. The small peak of plasma levels of OT detected in cows in which the CL had been removed at 0 min could be due to follicular oxytocin (Wathes et al., 1984) or luteal OT secreted in response to endogenous $PGF_{2\alpha}$ released as a consequence of manual manipulation of the ovary and/or uterus.

Luteal concentrations of OT did not change significantly over time, which concurs with the research of Abdelgadir et al. (1987) who found that tissue levels of OT did not change during *in vitro* $PGF_{2\alpha}$-induced secretion of this peptide. These investigators postulated that during incubation the CL replenished tissue levels of OT by conversion of the prohormone to the native OT molecule. This is a more likely explanation than *de novo* synthesis because Ivell et al. (1985) have shown that the CL has markedly decreased amounts of OT mRNA by day 8 of the cycle. In addition, Swann et al. (1984) demonstrated that *de novo* synthesis of OT required 12 h as indicated by incorporation of $[^{35}S]$ cysteine.

Upon cell stimulation, PKC translocates to the plasma membrane (Kraft and Anderson, 1983) where it can be activated by Ca^{2+}, diacylglycerol and phospholipids. We noted an increase in the PKC activity in the membrane fraction until 7.5 min after $PGF_{2\alpha}$ injection, which approximately corresponded to the time of peak OT release, but this increase was not significant. However, there was a significant positive correlation between membrane PKC activity and the level of OT in the systemic circulation. Because

PKC is known to translocate to the membrane upon stimulation, and membrane PKC activity was positively correlated with plasma OT in this study, the premise that $PGF_{2\alpha}$ acts through PKC to bring about the release of OT from the bovine CL is supported. There was no change in PKC activity in the cytosolic fraction. This was expected because PKC is involved in several cellular processes (Nishizuka, 1986) and, thus, a basal level of activity probably must be maintained in the cell.

Protein kinase C can also be activated by the Ca^{2+}-dependent protease, calpain, yielding a 50 kD fragment that is enzymatically active and independent of Ca^{2+}, diacylglycerol and phospholipids and that may have important physiological functions (Nishizuka, 1986). We investigated the possibility that $PGF_{2\alpha}$ may regulate the function of calpains and/or their endogenous inhibitor calpastatin and thereby affect the activity of PKC. To our knowledge, this is the first report of calpain activity in the bovine corpus luteum. m-Calpain was approximately twice as active as μ-calpain, but these activities were not affected by treatment with $PGF_{2\alpha}$. Activity of calpastatin increased slightly by 2 and 7 min after $PGF_{2\alpha}$ injection and was increased significantly by 30 min after injection. Although the increase in calpastatin activity by 2 min was not significant statistically, the increase in this calpain inhibitor may be biologically significant. It should be noted that calpastatin activity in the bovine CL was approximately 100 times greater than calpain activity (Fig 12 and 13: calpastatin activity was reported in terms of inhibition of m-calpain activity). Consequently, a small change in calpastatin activity could have a profound effect on calpain activity in this tissue.

Coupling previous reports with the present data it is possible to propose a dual role for $PGF_{2\alpha}$ in the regulation of PKC activity in the bovine corpus luteum which is consistent with our proposed model (Fig. 7). Prostaglandin $F_{2\alpha}$ first acts through the phosphoinositide cascade to cause the translocation of PKC to the membrane and its subsequent activation. A corollary effect of $PGF_{2\alpha}$ is to evoke an increase in calpastatin activity, which may result from an increase in total cellular concentration of this enzyme.

Activated calpastatin would in turn inhibit the proteolytic action of calpain and thereby increase the pool of the activated PKC holoenzyme at the membrane. The increased pool of activated PKC phosphorylates some unknown protein(s) that brings about the exocytosis of oxytocin.

The present research provides insight into the mechanisms of action of $PGF_{2\alpha}$-induced OT secretion during the life span of the corpus luteum. The regulation of PKC activity has been well characterized over the past decade; however, to our knowledge this is the first study evaluating the relationship between PKC, calpain and calpastatin in a hormonally-stimulated animal model. This *in vivo* approach provides a better understanding of the physiological function of these enzymes.

PROSTAGLANDIN $F_{2\alpha}$-INDUCED PHOSPHORYLATION OF THE MARCKS PROTEIN IN THE BOVINE CORPUS LUTEUM

Introduction

It has been well established that exposure of the fully developed ruminant corpus luteum to either exogenous (Louis et al., 1972) or endogenous (McCracken et al., 1972) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) results in luteolysis (Liehr et al., 1972; Rowson et al., 1972). In addition to causing luteolysis, this prostanoid causes the release of oxytocin (OT) from the large cells of the ruminant corpus luteum (Schallenberger et al., 1984; Walters et al., 1984; Lamsa et al., 1989; Flint et al., 1990). McCracken et al. (1984) proposed that a positive feedback loop exists between uterine $PGF_{2\alpha}$ and luteal oxytocin that causes the demise of the corpus luteum and the termination of the estrous cycle. Experimental evidence indicates that the action of $PGF_{2\alpha}$ is mediated by the activation of protein kinase C (PKC). Cosola-Smith et al. (1990) demonstrated that treatment of bovine luteal tissue slices with 12-O-tetradecanoylphorbol-13-acetate (TPA), a specific activator of protein kinase C, resulted in a release of OT similar to that caused by $PGF_{2\alpha}$. Furthermore, *in vivo* activation of this enzyme by $PGF_{2\alpha}$ has been shown to be highly correlated with luteal secretion of OT (Orwig et al., 1994b). While much is known about the intracellular biochemical changes that promote activation and inactivation of PKC, little is known about the substrates of this enzyme in the corpus luteum. Yet identification of these substrates is essential if progress is to be made toward fully understanding such phenomena as the ability of $PGF_{2\alpha}$ to induce luteal secretion of OT and associated events that result in luteolysis. One potential substrate for PKC phosphorylation in the bovine corpus luteum may be the 87 kD myristoylated alanine rich C kinase substrate (MARCKS).

Based on sequence analysis, bovine MARCKS is approximately 31 kD in size but migrates with an anomalously high molecular weight of 87 kD on SDS-polyacrylamide

gels (Blackshear, 1993). MARCKS is myristoylated post-translationally (Aderem et al., 1988) or co-translationally (James and Olson, 1989) and this modification is essential for membrane binding (George and Blackshear, 1992). However, the myristoylated form of MARCKS may also be found in the cytosol, and membrane association is not required for phosphorylation by PKC (Graff et al., 1989a). MARCKS binds calmodulin in a calcium dependent manner, but phosphorylation inhibits calmodulin binding (Graff et al., 1989c). Dephospho-MARCKS binds and cross-links actin filaments, but phosphorylation or calcium/calmodulin binding both decrease the ability of the protein to bind actin and abolish its ability to cross-link filaments (Hartwig et al., 1992). Clearly, phosphorylation of this PKC substrate can initiate a series of subcellular events that may be involved in the regulation of OT secretion from the bovine corpus luteum. Indeed, it has been demonstrated that phosphorylation of the MARCKS protein is correlated with the exocytosis of neurotransmitters (Robinson, 1991).

Several characteristics of the MARCKS protein make it a good candidate for $PGF_{2\alpha}$-induced phosphorylation by protein kinase C in the bovine corpus luteum. The MARCKS protein is a specific substrate for protein kinase C only (Blackshear et al., 1987; Graff et al., 1991), has a wide tissue distribution (Aderem, 1992; Blackshear, 1993) and has been detected and sequenced in the bovine brain (Stumpo et al., 1989). Furthermore, in the unphosphorylated state, this protein binds and cross-links actin filaments which have been shown to be involved in the exocytosis of granular contents from pituitary cells (Ostland et al., 1977; Kondo, 1987; Senda et al., 1989; Van de Moortele et al., 1991). Finally, the observation that $PGF_{2\alpha}$ results in the phosphorylation of the MARCKS protein in cultured MC-3T3-E1 osteoblasts (Quarles et al., 1993) suggests that $PGF_{2\alpha}$-induced activation of PKC in the bovine corpus luteum may also lead to the phosphorylation of this substrate.

Therefore, the present study was designed to determine whether the bovine corpus luteum contains the myristoylated alanine rich C kinase substrate. Providing that the

presence of MARCKS is established, experiments will be performed to determine whether the phosphorylation status of this PKC substrate is affected by treatment of luteal tissue slices with $PGF_{2\alpha}$ or TPA *in vitro*.

Materials and Methods

Beef heifers (12-16 months of age) were checked twice daily for behavioral estrus with a vasectomized bull. Normally cycling heifers were restrained on day 8 of the cycle (day of detected estrus = day 0 of the cycle) and 3 ml of 2% lidocaine were injected into the coccygeal spinal cord to induce caudal epidural anesthesia. Corpora lutea (CL) were removed per vaginum as modified from the procedure described by Casida (1959), dissected free of connective tissue, frozen in liquid nitrogen and stored at -80°C until analyses were performed.

Western blot analysis

Western blot analysis was performed on homogenates from day 8 bovine corpora lutea (0.5 g) to determine whether the MARCKS was expressed in this tissue. Bovine brain tissues were included as positive controls. Because MARCKS is a heat stable protein, samples were boiled for 10 min to remove all heat unstable proteins. Samples were analyzed for protein content using the Pierce BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard. Partially purified samples (100 μg protein) were separated by 7.5% SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970). Proteins were transferred onto a nitrocellulose membrane (0.45 μm, Amersham, Arlington Heights, IL) according to the methods of Towbin et al. (1979) in transfer buffer containing 20% methanol, 25 mM Tris-HCl and 192 mM glycine. After transfer, blots were washed with Tris-buffered saline (TBS) containing 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl and incubated at 25°C for 1.5 h with blocking solution (1% BSA-TBS). Membranes were then incubated with a

polyclonal antibody against bovine MARCKS (rabbit anti-peptide MARCKS antibody, 1:100 dilution in TBS containing 0.5% Tween-20, generously provided by Dr. Perry Blackshear, Duke University) for 2 h at 25°C, followed by incubation with horse radish peroxidase-conjugated goat anti-rabbit immunoglobin (1:2000 dilution, Bio-Rad, Richmond, CA) for 1 h at 25°C. Blots were washed five times with TBS containing 0.5% Tween-20. Protein bands were visualized using Chemiluminescence (Renaissance Western Blot Chemiluminescence Reagent Kit, Dupont, Boston, MA).

MARCKS phosphorylation

Corpora lutea were removed from three beef heifers at slaughter on Day 8 of the estrous cycle, immediately placed in ice cold medium and transported back to the laboratory. Corpora lutea were dissected free of adhering connective tissue, weighed and sliced (0.3 mm thickness).

Tissue incubations

Luteal slices were washed three times with Dulbecco's Modified Eagles Medium (D-MEM), 200 mg aliquots were added to each of seven tubes with 2 ml phosphate deficient D-MEM containing 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 30 μg/ml gentamicin and incubated for 1 h at 37°C. Following incubation in phosphate deficient medium, [^{32}P]-orthophosphate (200 μCi/ml) was added to each tube and the incubation continued for 45 min to allow incorporation of the radioactive label. Tubes were divided in duplicate to three treatment groups; ethanol vehicle (10 μl), prostaglandin $F_{2\alpha}$ (56 nM, in 10 μl ethanol) and TPA (0.2 μM, in 10 μl ethanol). A third TPA-treated tube was included for use as a negative control for the immunoprecipitation. Tissue slices were incubated with their specific treatments for 10 min and incubations were terminated

by placing samples on ice followed by centrifuging slices at 4°C. Slices were washed three times in ice cold medium to remove free label.

Subcellular fractionation

Tissue slices were homogenized in 1 ml buffer A (50 mM Tris-HCl, pH 8.3, 5 mM EDTA, 0.15 M NaCl and 5 μM microcystin) and centrifuged at 1000 x g for 10 min at 4° C to remove the nuclei. After centrifugation, the supernate was transferred to a new tube and centrifuged at 100,000 x g for 1 h at 4°C. The new supernate was designated the cytosolic fraction, the pellet was resuspended in buffer A containing 1% Nonidet P-40, incubated on ice for 30 min and centrifuged at 100,000 x g for 1 h at 4°C. The supernate from this centrifugation was designated the membrane fraction. Cytosolic and membrane fractions from all samples were heated in a boiling water bath for 10 min to precipitate all heat unstable proteins (MARCKS is heat stable). The extracts were then centrifuged at 15,000 x g to remove the precipitated proteins. The supernate was transferred to a new tube and protein determination was performed using the Pierce BCA protein assay (Pierce, Rockford, IL) to equalize the samples before immunoprecipitation.

Immunoprecipitation

Immunoprecipitation was performed essentially as described by Lobaugh and Blackshear (1990). Tissue extracts (equalized for protein content) were incubated with an anti-bovine MARCKS polyclonal antibody (1:100 dilution) overnight at 4°C. The third TPA-treated sample was treated with normal rabbit serum instead of the MARCKS antibody and served as a negative control. Samples were then incubated for 1 h at room temperature with protein G sepharose beads (Pierce, Rockford, IL). Beads were pelleted and the supernate removed. The pellet was washed five times with wash buffer (50 mM

Tris-HCl, pH 8.3, 0.15 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40 and 5 μM microcystin). Pelleted samples were resuspended in 60 μl wash buffer and 60 μl 2X SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 0.02% SDS, 0.05% β-ME and 0.0125% bromophenol blue). Samples were then subjected to 7.5% SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970), the gel was dried and autoradiography was performed by exposing the gel to Kodak X-OMAT film with intensifying screens. Phosphorylation of the MARCKS protein was quantitated for comparison between treatments using the model 1650 transmittance/reflectance scanning densitometer.

Because the MARCKS protein has been found in both membrane and cytosolic fractions and is known to translocate upon activation in some cell types, a Western blot was performed, as described above, on samples from the phosphorylation experiment to determine the level of the MARCKS protein present in each sample.

Results

Results of Western blot analysis using a polyclonal antipeptide antibody against bovine MARCKS revealed that the bovine corpus luteum does contain a positive signal for the PKC substrate at 87.4 kD (Fig. 14). A bovine brain sample was utilized as a positive control because MARCKS has previously been detected in this tissue (Stumpo et al., 1989). We consistently observe a non-specific band at 66.2 kD and have subsequently determined that this is bovine serum albumin (BSA, data not shown).

Compared to vehicle-treated controls, $PGF_{2\alpha}$ and TPA caused a significant increase in the phosphorylation of MARCKS, both in cytosolic and membrane fractions (Fig. 15, Top). Levels of phosphorylation between the membrane and cytosolic fractions from the same sample were not different. Because we expected TPA to cause the phosphorylation of MARCKS, a third TPA-treated sample was run as a negative control

for the immunoprecipitation procedure. This sample was precipitated with normal rabbit serum instead of the MARCKS antibody. No phospho-MARCKS was detected in this sample (data not shown), demonstrating that the phospho-protein observed in all other samples was specifically immunoprecipitated by the MARCKS antibody. Because MARCKS is known to translocate from the membrane to the cytosol upon phosphorylation in some cell types (Wang et al., 1989; Thelen et al., 1990), a Western blot experiment was performed to determine whether the change in phosphorylation was actually due to a translocation of the protein between cellular compartments. The upper band visible only in lanes 1,2 and 3 is MARCKS. The lower band visible in all lanes is non-specific binding to BSA. There was no effect of either treatment on the amount of MARCKS present in cytosolic samples (Fig. 15, bottom). However, MARCKS was undetectable in the membrane fraction. Based on the results of the phosphorylation experiment it is clear that the PKC substrate is present in this fraction, but it must be present only at low levels. Comparison of the non-specific BSA band between lanes provides evidence that an equal amount of protein was loaded onto each lane.

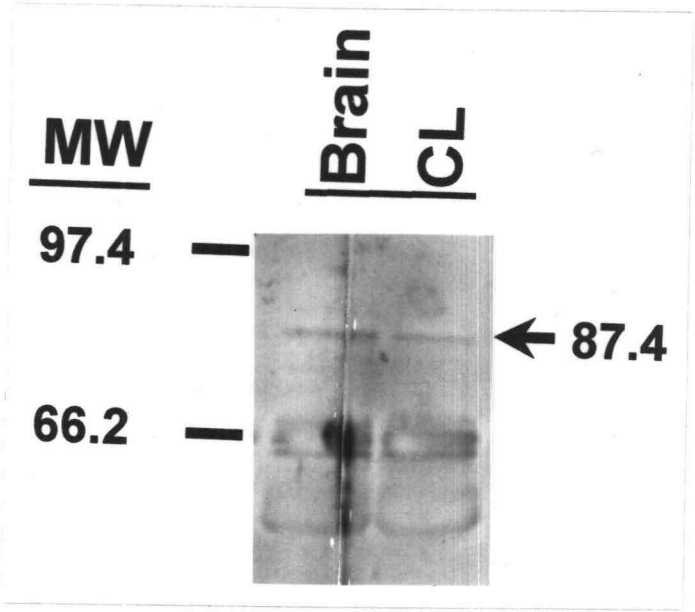

Fig. 14 Western blot demonstrating the presence of the MARCKS protein (designated by the arrow at 87.4 kD) in the bovine corpus luteum. Bovine brain was included as a positive control. Molecular weight standards (kD) are indicated.

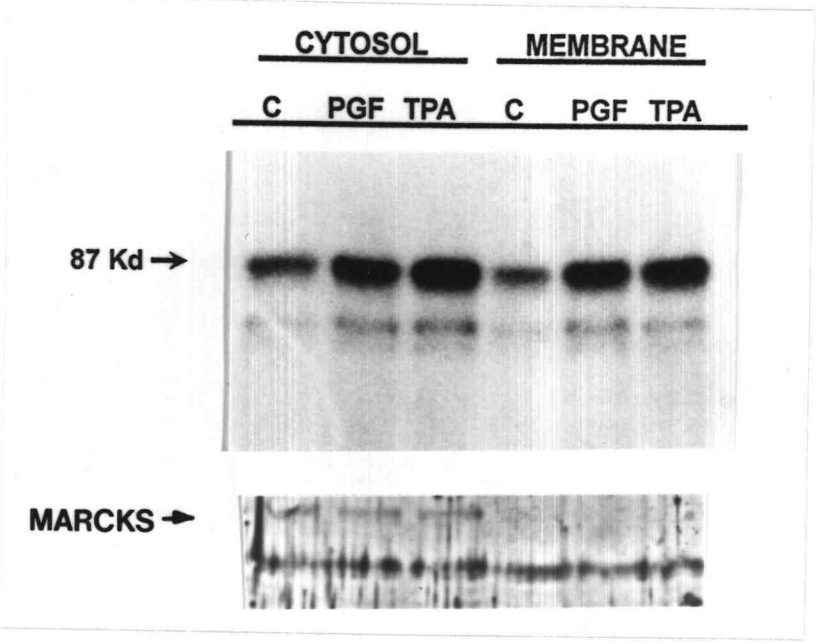

Fig. 15 Top panel: Phosphorylation experiment. Bovine tissue slices were treated *in vitro* with ethanol (control), $PGF_{2\alpha}$ or TPA in the presence of ^{32}P-orthophosphate. After incubation samples were separated into cytosolic and membrane fractions and phosphorylation of the MARCKS protein was visualized by autoradiography. Bottom panel: Western blot showing the level of the MARCKS protein in each lane from the phosphorylation experiment. The upper band visible only in lanes 1,2 and 3 is MARCKS. The lower band visible in all lanes is non-specific binding to BSA.

Discussion

Protein kinase C has been implicated as a second messenger in the $PGF_{2\alpha}$-induced secretion of oxytocin from the bovine corpus luteum (Cosola-Smith et al., 1990; Orwig et al., 1994b). However, the intracellular events distal to the $PGF_{2\alpha}$-induced activation of PKC, including possible substrates for this kinase have not been elucidated. We have demonstrated that a specific substrate for protein kinase C (MARCKS) is present in the bovine corpus luteum. This result was not unexpected because this protein has a wide tissue distribution and has been isolated and sequenced from the bovine brain (Stumpo et al., 1989). Bovine MARCKS migrated on an SDS-polyacrylamide gel with a M_r of 87.4 kD, which is in agreement with reports in the literature for this species (Blackshear, 1993). The specificity of the polyclonal MARCKS antibody was very good, but we consistently see strong non-specific binding to a 66.2 kD band which was subsequently determined to be bovine serum albumin (BSA).

Results of the phosphorylation experiment indicated that TPA caused an increase in the phosphorylation of the MARCKS protein in both cytosolic and membrane fractions compared to vehicle-treated controls. There was no difference between the level of phosphorylation in the membrane and cytosolic fractions. Because TPA is a known activator of protein kinase C, it was expected that this treatment would cause the phosphorylation of MARCKS. Treatment of bovine luteal tissue slices with a concentration of $PGF_{2\alpha}$ that has been shown to cause the secretion of oxytocin (Abdelgadir et al., 1987) also stimulated the phosphorylation of MARCKS in both cytosolic and membrane fractions, suggesting that this protein may be involved in the downstream regulation of OT secretion.

MARCKS is known to translocate from the membrane to cytosolic fraction upon phosphorylation in some cell types (Wang et al., 1989; Thelen et al., 1991). Therefore, a

Western blot analysis was performed on the samples from the phosphorylation experiment to determine whether the change observed in the phosphorylation status of MARCKS was actually due to a change in the cellular distribution of this protein. Results of the Western blot experiment revealed that there was no effect of treatment on the level of MARCKS present in the cytosolic fractions. However, the MARCKS protein was not detectable in membrane fractions. Yet, the level of MARCKS phosporylation in the membrane was similar to that in the cytosol. Therefore, it is suggested that only a small fraction of the cytosolic MARCKS protein is phosphorylated and the majority of the protein present in the membrane is phosphorylated. It is conceivable that the increase in phospho-MARCKS observed in the cytosol results from the translocation of this protein from the membrane and because phospho-MARCKS constitutes only a small fraction of the total cytosolic MARCKS population, this translocation was not detected at the level of the Western blot. This premise is supported by the data of Blackshear et al. (1985) who found that MARCKS is predominantly cytosolic in cultured fibroblasts and by the report of Wang et al. (1989) who demonstrated that MARCKS translocates from the membrane to the cytosol upon phosphorylation in isolated nerve terminals.

Protein kinase C exists as a family of at least 10 different, closely related isozymes (Nishizuka, 1992). We have previously demonstrated that the bovine corpus luteum contains the α and ε isozymes of protein kinase C (Orwig et al., 1994a). Blackshear et al. (1991) have demonstrated that a peptide analogous to the MARCKS phosphorylation domain is a substrate for the α, β, γ and ε isozymes of PKC. The ability of the other PKC isozymes to phosphorylate this peptide has not been determined. In contrast, Kiley et al. (1992) found that phorbol ester-induced phosphorylation of MARCKS was not affected by specific down-regulation of PKCε in GH_4C_1 pituitary tumor cells. However, phorbol ester-induced down-regulation of all PKC isozymes resulted in a 80% reduction in subsequent phorbol ester-stimulated phosphorylation of this protein. Collectively, these data indicate that phosphorylation of MARCKS, in this cell type, is a PKC-mediated event

that proceeds in the absence of PKCε. This does not preclude the possibility that the ε isozyme of protein kinase C phosphorylates MARCKS, it simply demonstrates that this isozyme is not an absolute requirement for MARCKS phosphorylation.

Phosphorylation of the MARCKS protein has been correlated with the exocytosis of neurotransmitters (Dunkley et al., 1986; Dunkley and Robinson, 1986). These investigators demonstrated that depolarization or phorbol ester-induced stimulation of intact synaptosomes, conditions that are known to cause the release of neurotransmitters, cause the phosphorylation of the MARCKS protein. In a 1992 review, Robinson provides a speculative model for the involvement of MARCKS in neurotransmitter release. This model suggests that MARCKS sequesters calmodulin (CaM) at the membrane of nerve terminals. Upon MARCKS phosphorylation, CaM is released from the membrane and becomes available to activate other proteins involved in the secretion of neuropeptides. In this regard, it is noteworthy that immunization against CaM prevented exocytosis from chromaffin cells (Kenigsberg and Trifaro, 1985).

We propose an alternative hypothesis for the $PGF_{2\alpha}$-induced secretion of OT, involving the actin binding capacity of the MARCKS protein. In nonmuscle cells actin exists in a dynamic equilibrium between filamentous and monomeric form (Bray et al., 1986). A dense, submembranous network of actin filaments exists in several secretory cell types and disruption of this network occurs under exocytotic stimulus (Schlessinger and Geiger, 1981; Sobue et al., 1988). Orci et al. (1972) demonstrated that when the actin filament rich "cell web" in pancreatic β-cells is disrupted by cytochalasin B, glucose-stimulated insulin secretion increases. Dephosphorylated MARCKS binds and cross-links actin filaments (Hartwig et al., 1992) and could potentially provide stability to the "cell web" described by Orci. Furthermore, phospho-MARCKS does not cross-link actin filaments (Hartwig et al., 1992), and conditions that cause MARCKS phosphorylation are correlated with extensive rearrangement of the actin cytoskeleton (Aderem, 1992).

We propose that in the unstimulated state, the large cells of the corpus luteum have a submembranous actin network that is stabilized by MARCKS cross-linking of actin filaments. The dense nature of this network prevents the movement of oxytocin-laden secretory granules to the membrane. Prostaglandin $F_{2\alpha}$-induced activation of protein kinase C, and hence, phosphorylation of MARCKS results in the disruption of the submembranous cytoskeleton and allows the movement of secretory granules to the membrane and subsequent exocytosis (Fig. 16).

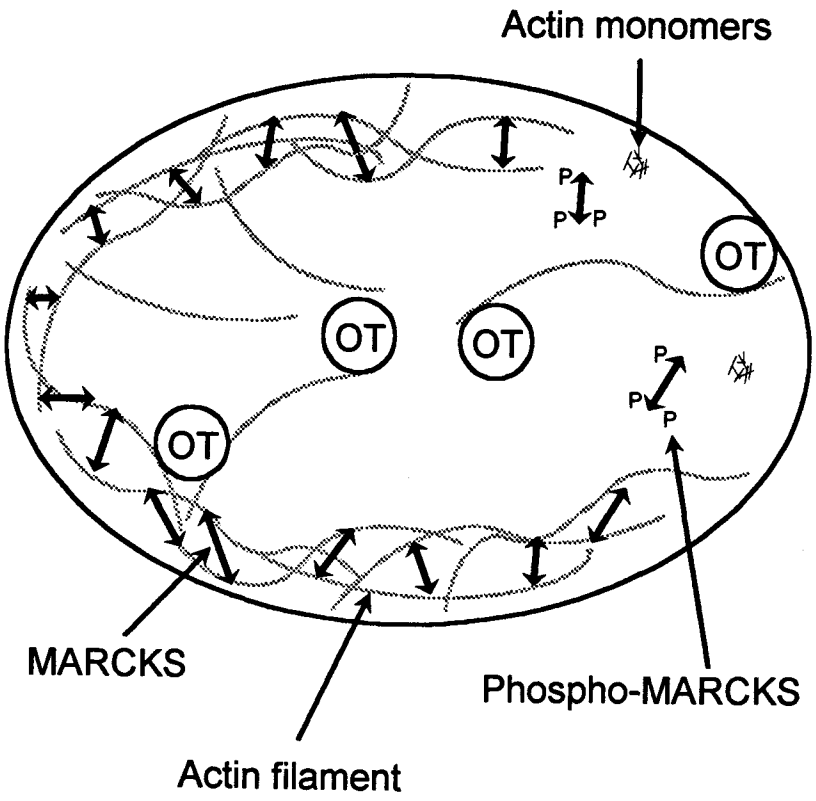

Fig. 16 Proposed model for the involvement of MARCKS in the $PGF_{2\alpha}$-induced secretion of oxytocin (OT) from the bovine corpus luteum. Submembranous actin filament network is extensively cross-linked by the unphosphorylated MARCKS protein and inhibits the movement of oxytocin-containing secretory granules to the membrane. Prostaglandin $F_{2\alpha}$ causes the phosphorylation of MARCKS and subsequent disruption of the actin network; thus allowing the exocytosis of oxytocin.

Clearly, additional experiments will be required to substantiate this hypothesis, but the data presented herein, provide the groundwork for future research related to the regulation of exocytotic processes in the bovine corpus luteum.

GENERAL DISCUSSION

Results of *in vitro* experiments implicated protein kinase C as a second messenger in the prostaglandin $F_{2\alpha}$-induced secretion of oxytocin from the bovine corpus luteum. However, it was not known which PKC isozymes were present in this tissue. Elucidation of the isozymes of protein kinase C present in the bovine corpus luteum, as well as their subcellular distribution, was deemed essential in order to provide insight into the possible role for this kinase in oxytocin secretion. Because several isozymes of protein kinase C are known to translocate from the cytosol to the membrane during activation, the *in vivo* effect of $PGF_{2\alpha}$ on the subcellular distribution and activity of this kinase was evaluated. Review of the regulation of PKC activity revealed that the calcium activated neutral proteases, calpains, and their endogenous inhibitor calpastatin may be important in the activation or inactivation of this enzyme. The *in vivo* effect of $PGF_{2\alpha}$ on these activities was also determined to be important. Finally, nothing was known about the intracellular molecular events beyond the activation of protein kinase C that bring about the $PGF_{2\alpha}$-induced secretion of oxytocin. In this regard, a myristoylated alanine rich C kinase substrate is a specific receptor for phosphorylation by protein kinase C in several species and tissue types, including the bovine brain. The efficacy of this protein as a substrate for phosphorylation by PKC in the bovine corpus luteum was evaluated.

Research was conducted which demostrated that the bovine corpus luteum contains the α and ε isozymes of protein kinase C, but not the β, γ, δ, or ζ isoforms. The α isozyme was localized predominantly in the cytosol, while the ε isozyme was located mostly in the membrane fraction. While the roles of PKCα and ε in the bovine CL are not known, both isozymes have been implicated in exocytotic events in pituitary tumor cells (Naor et al., 1989; Akita et al., 1990). However, these isozymes are subjected to different modes of activation, regulation, and substrate specificity, allowing for diverse cellular responses. Because the CL is composed of both large and small luteal cells, it remains to

be determined which isozyme(s) is expressed in the large cells of the corpus luteum that contain oxytocin.

Results of a subsequent experiment demonstrated that *in vivo* administration of $PGF_{2\alpha}$ to beef heifers on day 8 of the estrous cycle resulted in a peak release of oxytocin into the systemic circulation that occurred between 1.5 and 10 min after injection of the luteolysin. Those animals from which the CL was removed before the injection of $PGF_{2\alpha}$ did not exhibit a peak release of OT that was significantly greater than baseline, suggesting that most of the OT release observed was from the corpus luteum and not of neurohypophyseal origin. This observation is supported by the data of Schams et al. (1985), who reported that the posterior pituitary released only minute quantities of oxytocin after prostaglandin injection. Luteal concentrations of OT did not change significantly over time after $PGF_{2\alpha}$ injection, which concurs with the research of Abdelgadir et al. (1987), who found that tissue levels of OT did not change during *in vitro* $PGF_{2\alpha}$-induced release of this peptide.

Membrane associated protein kinase C activity increased until 7.5 min after $PGF_{2\alpha}$ injection, but this increase was not significant. However, there was a significant positive correlation between membrane PKC activity and the level of OT in the systemic circulation. Because PKC is known to translocate to the membrane upon activation (Kraft and Anderson, 1983) and membrane associated PKC activity was positively correlated with plasma OT in this study, the premise that $PGF_{2\alpha}$ acts through PKC to bring about the release of OT from the bovine CL is supported. Activity of protein kinase C in the cytosol did not change over time after treatment with $PGF_{2\alpha}$. This was expected because PKC is involved in several cellular processes (Nishizuka, 1986), and thus, a basal level of activity probably must be maintained in the cell.

Calpain activities were not affected by treatment with $PGF_{2\alpha}$, but calpastatin activity increased slightly by 2 and 7.5 min after injection and significantly by 30 min after injection. Calpains hydrolyze the membrane bound PKC holoenzyme to a lower molecular

weight, constitutively active form of the enzyme (Melloni et al., 1986; Nishizuka, 1986). It is suggested herein that $PGF_{2\alpha}$ causes increased calpastatin activity, which results in inhibition of cellular calpain activity and selectively increases the pool of the PKC holoenzyme in the membrane. The active PKC holoenzyme phosphorylates some protein or proteins that bring about the exocytosis of oxytocin either directly or indirectly (Fig. 17).

The regulation of protein kinase C activity has been studied extensively over the past decade in cultured cell and cell free experiments. To our knowledge, this is the first study evaluating the interrelationships between PKC, calpains and calpastatin in a hormonally-stimulated *in vivo* model.

The ability of the myristoylated alanine rich C kinase substrate (MARCKS) to serve as a substrate for $PGF_{2\alpha}$-activated protein kinase C was evaluated. It was demonstrated that the bovine corpus luteum does indeed contain this PKC substrate and it migrated on an SDS-polyacrylamide gel with an apparent molecular weight of 87.4 kD, which is consistent with reports in the literature for this species (Blackshear, 1993). As expected, *in vitro* treatment of bovine luteal tissue slices with the PKC activating phorbol ester, TPA, resulted in the increased phosphorylation of this protein. Treatment with a concentration of $PGF_{2\alpha}$ that has been shown to cause the release of OT from bovine luteal tissue slices (Abdelgadir et al., 1987) also caused the phosphorylation of the MARCKS protein in both the cytosolic and membrane fraction compared with vehicle-treated controls. It is suggested that the majority of the membrane associated MARCKS protein is phosphorylated and the increase in the phosphorylation status of cytosolic MARCKS may result from translocation of the protein from the membrane. However, this translocation event could not be demonstrated by Western blot analysis because the low level of MARCKS present in the membrane fraction was not detectable by this method. It should be noted that MARCKS has been implicated in the secretion of neurotransmitters

(Robinson, 1991), and therefore, is a good candidate for the PKC substrate that regulates the secretion of OT from the bovine corpus luteum.

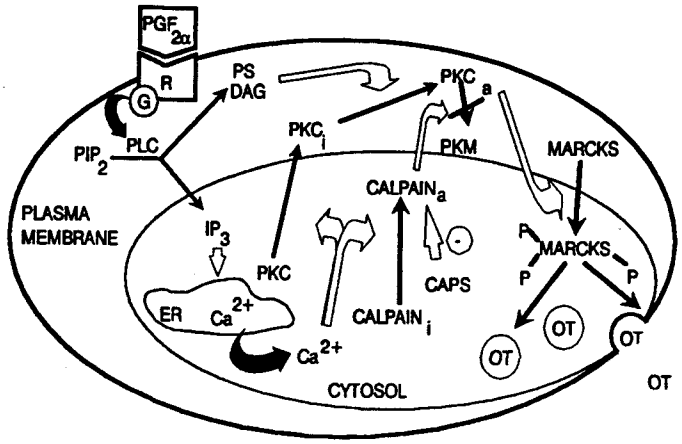

Fig. 17 Functional interrelationships between protein kinase C (PKC), calpains, calpastatin and the myristoylated alanine rich C kinase substrate (MARCKS) during $PGF_{2\alpha}$-induced secretion of oxytocin. See fig. 7 for abbreviations.

We have proposed a model (Fig. 16) for the involvement of MARCKS in the $PGF_{2\alpha}$-induced secretion of oxytocin from the bovine corpus luteum. This model suggests that in the unstimulated state, the large cells of the corpus luteum have a submembranous actin network that is extensively cross-linked by the MARCKS protein. The dense nature of this actin network prevents the movement of OT-laden secretory granules to the plasma membrane. Prostaglandin $F_{2\alpha}$-induced activation of protein kinase C, and hence, phosphorylation of MARCKS results in the disruption of this submembranous actin network and allows the movement of secretory granules to the membrane and subsequent exocytosis.

This model is merely speculative and further research will be required to prove or disprove its validity. Potential experiments that may prove useful in testing the proposed model include staining large luteal cells for actin to determine whether this cell type does

indeed contain a subcortical actin network, and if so, whether the integrity of the network is affected by treatment with $PGF_{2\alpha}$. It will also be necessary to characterize the association of MARCKS with actin in the bovine corpus luteum and determine whether that relationship is affected by treatment with $PGF_{2\alpha}$. Protein cross-linking experiments may provide information regarding the interaction between MARCKS and actin. The proposed model provides the groundwork for future research related to the regulation of oxytocin secretion from the bovine corpus luteum. In fact, the proposed model may prove useful in the study of exocytosis of peptide or protein hormones from a variety of other endocrine tissues.

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APPENDIX

UNIQUE METABOLITES OF EICOSAPENTAENOIC ACID INTERFERE WITH CORPUS LUTEUM FUNCTION IN THE EWE

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Abstract

Experiments were conducted to determine the *in vivo* and *in vitro* effects of metabolites of eicosapentaenoic acid on ovine luteal function. Injection of 750 μg methyl eicosapentaenoic acid (EPA) or methyl 12(*R*),13(*S*)-dihydroxyeicosapentaenoic acid (12,13-diHEPE) into the ovarian artery of ewes on day 10 of the estrous cycle caused a reduction in serum concentrations of progesterone by 48 h post-treatment compared with levels of this steroid in arachidic acid-treated controls ($p<0.005$). Although mean serum concentrations of progesterone in methyl EPA-treated ewes during the remainder of the cycle did not differ from those in control ewes, levels in methyl 12,13-diHEPE-treated ewes remained significantly suppressed. Duration of the estrous cycle did not differ among treatment groups ($p>0.05$), but more of the methyl 12,13-diHEPE-treated animals (3/5) had exhibited estrus within 3 days after injection than methyl EPA-treated (1/5) or control ewes (0/5). Slices of corpus luteum removed from ewes on day 10 of the estrous cycle were incubated with arachidic acid (controls), 12,13-diHEPE or docosatetraenoic acid (DTA). Regardless of fatty acid treatment, all tissues retained the ability to produce

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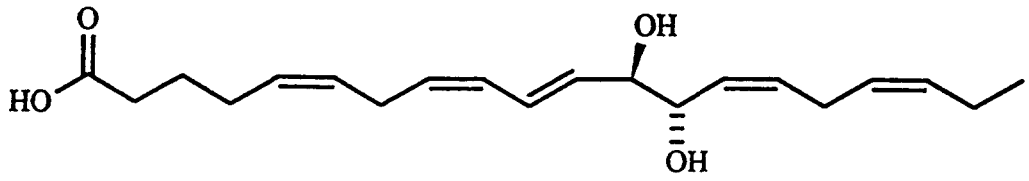
[2]Technical paper 9989 from the Oregon Agricultural Experiment Station.

basal levels of progesterone during subsequent incubation. Luteal slices previously exposed to arachidic acid or DTA exhibited an increase in progesterone production in response to subsequent treatment with LH ($p<0.05$). In contrast, luteal slices incubated with 12,13-diHEPE did not respond to LH with a significant increase in production of this steroid above that observed in controls. All tissues displayed a marked increase in progesterone synthesis upon treatment with 8-Br-cAMP relative to incubation of tissue alone ($p<0.001$). Subcellular distribution of [^{14}C]-12,13-diHEPE in luteal cells after incubation revealed that the majority of the fatty acid was associated with the plasma membrane. These data suggest that metabolites of eicosapentaenoic acid with hydroxyl groups on adjacent carbon atoms interfere with luteal function in the ewe, perhaps in part by altering luteal response to LH.

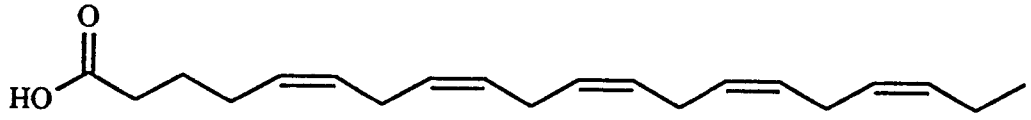
Introduction

Metabolism of arachidonic acid via the cyclooxygenase pathway yields products that affect the function of the corpus luteum (CL). One such metabolite, prostaglandin (PG) $F_{2\alpha}$ is well documented to be luteolytic in several mammalian species (1), while others, PGE_2 and PGI_2, reportedly prolong luteal life span in cows (2,3) and ewes (4,5) and increase *in vitro* basal progesterone production by bovine luteal cells (6). In contrast, much less is known about the significance or possible function of long-chain metabolites of polyunsaturated fatty acids. While prostaglandins act by binding to membrane receptors to provoke changes in intracellular second messengers, it is conceivable that long-chain, polyunsaturated fatty acids become integral components of the plasma and(or) intracellular membranes and thereby interfere with normal cell function. In this regard, Zelinski et al. (7) found that levels of docosatetraenoic acid (22:4) and docosapentaenoic acid (22:5) were significantly greater in plasma membranes of regressing CL in nonpregnant ewes than in CL of pregnant ewes on day 15 after estrus. In addition, Milvae et al. (8) found

that *in vitro* exposure of bovine luteal cells to 5-hydroxyeicosatetraenoic acid caused a reduction in basal and LH-stimulated progesterone production.



12(*R*),13(*S*)-diHEPE



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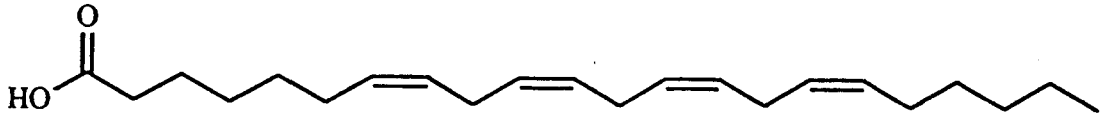
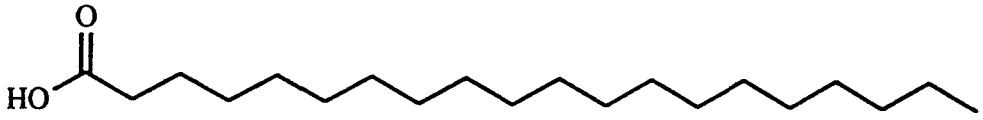


Docosatetraenoic acid



Arachidic acid

Recently Solem et al. (9) and Lumin and Falck (10) reported the isolation of 12(R), 13(S)-dihydroxyeicosa-5(Z), 8(Z), 10(E), 14(Z), 17(Z)-pentaenoic acid (diHEPE)

from the marine red alga *Farlowia mollis*. This fatty acid was shown to be a weak stimulator of superoxide anion production by human neutrophils, an activity that may also be important in natural luteal regression. Indeed, Riley and Behrman (11) demonstrated that luteal hydrogen peroxide levels rapidly increase *in vivo* with onset of CL regression.

The present study was conducted to examine the *in vivo* and *in vitro* effects of 12,13-diHEPE, eicosapentaenoic acid (EPA), a closely related fatty acid of similar chain length and unsaturation, and docosatetraenoic acid (DTA) on ovine luteal function.

Methods

Isolation of methyl 12(R),13(S)-diHEPE

An extract of *G. lemaneiformis* (13 g, 1 July 1988, Cape Perpetua, Oregon) was methylated, peracetylated and chromatographed to provide a mixture of methyl 12(*R*),13(*S*)-diacetoxyeicosapentaenoate, essentially as previously described by Jiang and Gerwick (12). This was saponified [5% potassium hydroxide (KOH)/methanol (MeOH) (1:3), 50°C, 15 min], products extracted [methylene chloride (CH_2Cl_2), 120 ml] from the reduced and acidified (pH 6) extract, methylated (CH_2N_2), and rechromatographed on HPLC [35% ethyl acetate (EtOAc)/hexane (hex), Alltech RSil 10μ 50 cm x 10 mm] to yield 58.3 mg of pure methyl 12(*R*),13(*S*)-diHEPE. An extract (3 g) of a collection of *Farlowia mollis* (Shell Beach, CA, 1 Aug 1988) was vacuum chromatographed over silica gel, fractions containing 12(*R*),13(*S*)-diHEPE methylated (CH_2N_2), and then purified by HPLC (35% EtOAc/hex, Alltech RSil 10μ 50 cm x 10 mm) to also provide pure methyl 12(*R*),13(*S*)-diHEPE (56.4 mg, 9). Both samples were characterized by ^{1}H-NMR and subsequently combined to yield 108 mg of pure sample.

Enzymatic formation of 12(R),13(S)-diHEPE

Dihydroxyeicosapentaenoic acid was formed enzymatically as described by Moghaddam and Gerwick (13). Briefly, eicosapentaenoic acid (90 mg) was incubated with 46 g *G. lemaneiformis* acetone powder, prepared as previously described (14), in 500 ml of 0.1 M K_2HPO_4 buffer (pH 7.4) for 60 min (RT). The reaction was terminated by filtration through cheese cloth and the pH adjusted to 4.0 with 5% HCl. This was repetitively extracted with chloroform ($CHCl_3$) (3 x 300 ml) which, following *in vacuo* removal of solvent, gave 23.5 mg of green oil. The oil was applied to a preparative TLC plate, developed in diethyl ether/hexane/acetic acid (64:35:1), and a broad band R_f 0.05-0.2 removed and the compound eluted with diethyl ether (Et_2O). The 12(*R*),13(*S*)-diHEPE was further purified by HPLC [2 x 4.1 mm x 250 mm 10μ Versapak, 7% isopropyl alcohol (IPA)/hex with 0.1% acetic acid] to yield 1.1 mg. This material was subjected to ^{1}H-NMR and TLC, and characteristics compared with our earlier work in this structural class.

Enzymatic formation of [1-^{14}C]-12(R),13(S)-diHEPE

[1-^{14}C]-eicosapentaenoic acid (New England Nuclear Research, 1.85 Bq, 1.5 GBq/mmol, 0.38 mg) was added to an equal mass of unlabeled eicosapentaenoic acid and then incubated in 160 ml of acetone powder supernatant [formed by stirring 6.7 g acetone powder in 170 ml 0.1 M K_2PO_4 buffer (pH 7.4) for 60 min and then centrifuged at 12,000 x *g* for 30 min]. After 60 min, the reaction was terminated with the addition of 150 ml MeOH. The lipids were then salted out into 3 x 250 ml $CHCl_3$ which was then reduced under vacuum to yield a yellowish-green oil. This material was applied in Et_2O to a TLC plate, developed in Et_2O-hex-HOAc (64:35:1), and a band (R_f 0.05-0.2) removed and the

compound eluted with Et_2O. This was further purified by HPLC (2 x 4.1 mm x 250 mm 10μ Versapak, 7% IPA/hex with 0.1% acetic acid) to provide a radioactive yield of 0.8%.

Animals

Twenty-six mature Polypay ewes were checked twice daily for behavioral estrus with a vasectomized ram. Upon completion of two normal estrous cycles, ewes were assigned to one of three experiments. For all experiments, ewes were laparotomized on day 10 of the cycle (day of detected estrus = day 0). Animals were initially anesthetized by iv injection of sodium thiamylal (Biotal 2%) and anesthesia was maintained by halothane-oxygen inhalation. All surgical procedures were carried out under aseptic conditions.

Experiments

Experiment 1 was conducted to determine whether administration of methyl 12,13-diHEPE and methyl EPA would alter luteal function in ewes. Fifteen ewes were assigned randomly to one of three groups (n=5 each) and were laparotomized as described above. Ewes with corpora lutea in both ovaries were unilaterally ovariectomized, while those with CL in only one ovary were allowed to remain intact. The treatment each group of ewes received at laparotomy was as follows: group 1, methyl 12,13-diHEPE; group 2, methyl EPA; and group 3, controls, methyl arachidic acid. The dosage of each fatty acid was 750 μg in 0.1 ml of dimethylsulfoxide (DMSO) and was injected into an artery supplying the ovary bearing a CL. Methyl eicosapentaenoic acid was chosen to be administered because this fatty acid is similar to methyl 12,13-diHEPE in degree of unsaturation and chain length. Control ewes were given methyl arachidic acid, a saturated fatty acid with the same chain length as methyl 12,13-diHEPE and methyl EPA. Blood samples were

collected via jugular venipuncture just prior to treatment and on alternate days thereafter until ewes returned to estrus. Resulting sera were analyzed for progesterone by radioimmunoassay. Effect of treatments on duration of the estrous cycles were also recorded.

Results of experiment 1 suggested that methyl 12,13-diHEPE altered ovine luteal function *in vivo* and previous research (7) demonstrated that the concentration of docosatetraenoic acid (DTA) increased in plasma membranes of regressing CL of ewes. Therefore, experiment 2 was conducted to evaluate the *in vitro* effects of 12,13-diHEPE and DTA on LH and cAMP-stimulated progesterone production by luteal tissue. Five pairs of ewes (n=10 total) were laparotomized on day 10 of the estrous cycle. Corpora lutea enucleated from the ovaries of each pair of ewes were immediately placed in cold Ham's F-12 supplemented as described by Slayden and Stormshak (15). Corpora lutea were dissected free of adhering connective tissue, weighed, and sliced (0.3 mm thickness). Luteal slices from two ewes were pooled, washed three times with cold Ham's F-12 and 50 mg aliquots were added to each of 26 tubes containing 1 ml of Ham's F-12 medium maintained at 4°C. Cold absolute ethanol was immediately added to two tubes, designated unincubated controls, to preclude further progesterone synthesis. The remaining 24 tubes were divided randomly into three sets of eight and received the following fatty acids dissolved in 10 µl DMSO: set #1, controls, 1 µM arachidic acid; set #2, 1 µM 12,13-diHEPE and set #3, 1 µM docosatetraenoic acid. Concentrations of fatty acids were low enough to avoid nonspecific fatty acid effects and were below those reported to cause disruption of luteal cell membranes (16). Samples were incubated for 90 min in a Dubnoff shaking incubator at 37°C under an atmosphere of 95% O_2 - 5% CO_2. Following incubation, medium was removed and tissue slices were washed twice with fresh medium. Two samples from each of sets 1, 2 and 3 (6 tubes total), were chosen as unincubated controls for the next incubation. These samples were suspended in 1 ml incubation medium and 2 ml cold ethanol were added to preclude further synthesis of progesterone.

All remaining tubes were resuspended in 1 ml fresh medium. Two samples from each of the three sets received vehicle alone (10 µl saline), two received 50 ng ovine LH (in 10 µl saline), and two samples were treated with 8-Br-cAMP (in 10 µl saline) to result in a final concentration of 20 mM 8-Br-cAMP. Samples were incubated for 2 h under the same conditions as described above and incubation was terminated by addition of 2 ml of cold absolute ethanol to tissue and medium. Samples were stored at -20°C until analyzed for progesterone.

The objective of experiment 3 was to determine the subcellular distribution of 12,13-diHEPE and thus provide insight regarding its mode of action in altering luteal cell function. Corpora lutea were recovered from one ewe via laparotomy on day 10 of the estrous cycle. Tissue was immediately placed in medium (4°C), transported to the laboratory and processed as described for experiment 2. Luteal slices (400 mg) were added to a flask containing 3 ml of medium with 1 µg/ml of [1-^{14}C]-12,13-diHEPE (0.2 µCi) and incubated for 2 h under 95% O_2 - 5% CO_2. After incubation, tissue was washed three times with medium, resuspended in 3 ml of saline and stored at -80°C until analysis was performed on subcellular fractions.

Subcellular fractionation of luteal tissue was accomplished by differential and discontinuous sucrose gradient centrifugation as described by Gospodarowicz (17) and Bramley and Ryan (18). All supernatants and tissue pellets were then gently resuspended in buffer using a Dounce homogenizer (size A) and recentrifuged at the same speed and time to ensure maximal sedimentation of each subcellular organelle. It has been demonstrated that ovine luteal subcellular fractions separated by this technique are relatively pure and free of contamination by other fractions (7). Aliquots (100 µl) of each subcellular fraction suspended in buffer containing 25 mM tris-base (pH 7.5) and 1 mM $CaCl_2$, were analyzed for protein using the Pierce Protein Assay Kit (Rockford, IL). Incorporation of radioactivity was determined for the purified subcellular fractions by use of a Beckman LS 6000 ß-scintillation counter.

Radioimmunoassays

All blood samples (10 ml) were allowed to clot at room temperature, then stored at 4°C overnight. Sera were separated by centrifugation at 1100 x *g* for 10 min at 4°C and stored at -20°C until assayed for progesterone. Samples were analyzed in duplicate after hexane:benzene (2:1) extraction. [1,2,6,7-^{3}H] progesterone (12 x 10^3 dpm; New England Nuclear; Net-381) was added to a third tube containing an aliquot of the sample to determine and correct for procedural loss due to extraction. Efficiency of extraction of progesterone from sera was 94.2 ± 0.2%. Progesterone radioimmunoassays were performed using anti-progesterone-11-BSA (provided by Dr. Gordon Niswender, Colorado State University). The sensitivity of the assay was 10 pg/assay tube (100 µl). Intra- and interassay coefficients of variation for analysis of serum samples in Exp. 1 were 8.7 and 5.8%, respectively.

Tissue samples (tissue plus medium) were prepared for progesterone assay as described by Koligian and Stormshak (19). Before homogenization, [^{3}H]-progesterone (45 x 10^3 dpm) was added to each sample to determine procedural loss. Addition of [^{3}H]-progesterone to tissue samples increased total progesterone in tissue extractant by 3.5 pg/ml, which was below the limit of detection of the assay. Tissue and medium in absolute ethanol were homogenized with an additional 6 ml absolute ethanol in a Duall 24 ground glass homogenizer. Samples were filtered through Whatman no. 1 filter paper with 15 ml absolute ethanol. Filtered samples were evaporated under vacuum at 45°C until dry, resuspended in 3 ml distilled water and extracted with 20 ml benzene:hexane (2:1). A 1 ml fraction of extractant was used to determine procedural loss. The extraction efficiency was 92 ± 6.3% (n=130). Intra- and interassay coefficients of variation for assay of samples in Exp. 2 were 10.5 and 11.5%, respectively.

Statistics

Data on serum concentrations of progesterone in control, methyl 12,13-diHEPE and methyl EPA-treated ewes for a given day were analyzed separately from data acquired for other sampling times by use of one-way analysis of variance. This was necessary because some ewes treated with methyl 12,13-diHEPE exhibited estrus within 72 h after treatment, thus numbers of ewes were not constant among groups for each of the days of sampling that followed treatment.

Luteal concentrations of progesterone in tissue after the initial 90 min incubation with fatty acids were considered as unincubated controls and were subtracted from the levels of steroid present after incubation in the presence or absence of LH and 8-Br-cAMP. The resulting data were analyzed as for an experiment of randomized block design by analysis of variance. Differences among means were evaluated for significance by the F-protected least significant difference test.

Results

A single injection of 750 μg of methyl 12,13-diHEPE or methyl EPA into the ovarian artery of ewes on day 10 of the estrous cycle caused mean serum concentrations of progesterone to be significantly lower than that of control ewes during the ensuing 4 days of the cycle ($p<0.005$, fig. 1). Similarly, serum concentrations of progesterone in methyl 12,13-diHEPE-treated ewes were lower than those of ewes treated with methyl EPA ($p<0.05$). However, by the fourth day post-treatment, mean serum concentrations of progesterone in methyl EPA-treated ewes did not differ from those in control ewes.

Although duration of the estrous cycle did not differ significantly among treatment groups ($p>0.05$, data not shown), more of the methyl 12,13-diHEPE treated animals (3/5)

had exhibited estrus within 3 days after injection than methyl EPA-treated (1/5) or control ewes (0/5).

In experiment 2 the mean luteal concentration of progesterone prior to initial incubations with the fatty acids was 3.16 ± 0.38 μg/mg luteal tissue. Luteal concentrations of progesterone after 90 min incubation with arachidic acid, 12,13-diHEPE, or DTA were 5.14 ± 0.42, 4.51 ± 0.28 and 4.69 ± 0.48 μg/mg luteal tissue, respectively, and did not differ ($p>0.05$). Incubation of luteal tissue with these fatty acids did not affect progesterone production in response to the subsequent 2 h incubation in the absence of LH or cAMP ($p>0.05$). Relative to quantity of steroid produced in the absence of gonadotropin, addition of LH to the medium increased progesterone production by luteal tissue regardless of whether previously exposed to arachidic acid, 12,13-diHEPE or EPA (fig. 2). However, the increase in progesterone production due to LH was significant only in control and DTA-treated luteal tissue ($p<0.05$, respectively). Previous exposure of luteal tissue to arachidic acid, diHEPE or DTA did not affect its *in vitro* ability to respond to 8-Br-cAMP. Irrespective of the fatty acid to which the tissue had been exposed, treatment with 8-Br-cAMP caused a marked increase ($p<0.001$) in progesterone synthesis relative to that of the incubated control tissue.

Radiolabeled 12,13-diHEPE associated with various subcellular fractions of luteal tissue incubated in the presence of [1-^{14}C]-12,13-diHEPE for 2 h (Exp. 3) is depicted in Fig. 3. Only 0.6% of the original 0.2 μCi [1-^{14}C]-12,13-diHEPE added was recovered in subcellular fractions. The majority of the recovered radioisotope was associated with the plasma membrane (49.0%) with comparatively lesser amounts in the endoplasmic reticulum (17.8%), mitochondria (9.8%), nuclei (9.8%), microsomes (8.0%), and cytosol (5.5%).

Discussion

Based upon the observed reduction in serum concentrations of progesterone and duration of the estrous cycle it is apparent that injection of methyl 12,13-diHEPE directly into the ovarian artery was more effective than methyl EPA in altering luteal function in ewes.

Responses of luteal slices to LH and 8-Br-cAMP after *in vitro* exposure to 12,13-diHEPE and docosatetraenoic acid provided further insight regarding the site of action of these unsaturated fatty acids. Kaltenbach et al. (20) have demonstrated that LH enhances secretion of progesterone from ovine luteal tissue *in vitro*. Cyclic AMP has been shown to activate cholesterol esterase and therefore appears to be an important point of regulation of steroidogenesis (21). Data from the *in vitro* study suggest that the suppressive effect of 12,13-diHEPE on luteal function may be exerted at the level of the plasma membrane and not at a distal intracellular site beyond the production of cAMP. This proposed site of action of 12,13-diHEPE is supported by results of the study in which luteal tissue was incubated with this labeled fatty acid. These latter data revealed that the majority of the isotopically labeled fatty acid was associated with the plasma membrane of luteal cells. However, the possibility that 12,13-diHEPE also acts at other sites within the cell cannot be entirely excluded.

Arachidic acid, 12,13-diHEPE and EPA are of equal chain length but 12,13-diHEPE and EPA are polyunsaturated and characterized by the presence of five double bonds. Thus, it appears that the ability of 12,13-diHEPE and EPA to attenuate progesterone secretion and(or) cause premature luteal regression was dependent in part upon their unsaturation. However, the greater effectiveness of 12,13-diHEPE compared with EPA in suppressing luteal function may be attributed to either the position of the third double bond and(or) the hydroxyl groups located at carbons 12 and 13 of the molecule. The observed *in vivo* effects of methyl 12,13-diHEPE and methyl EPA on

ovine corpus luteum function are similar to those evoked by methyl sterculic acid, a 19 carbon cyclopropenoid fatty acid of plant origin. Administration of this fatty acid to nonpregnant ewes reduced serum concentrations of progesterone and caused premature luteal regression (15). Collectively, these data suggest that structural features of long chain fatty acids beyond the degree of unsaturation dictate their ability to impair the function of the luteal cell. In this regard, the observed lack of effect of docosatetraenoic acid on luteal progesterone production *in vitro* may be attributed to the fact that this fatty acid has only four double bonds and no additional side groups.

The existing data suggest that 12,13-diHEPE interferes with the ability of the luteal cell to respond to LH. The precise mode of action of 12,13-diHEPE in accomplishing this feat is unknown. However, it is speculated that 12,13-diHEPE may interact directly with the LH receptor making it inaccessible to the ligand. Cortell et al. (22) reported that methyl sterculic acid suppressed ovine luteal function by interfering with the synthesis and(or) recycling of the LH receptors. Alternatively, 12,13-diHEPE may be incorporated into the luteal plasma membrane, resulting in reduced membrane fluidity. Such a change in membrane characteristics could hinder LH receptor associated G-protein mobility and hence subsequent activation of adenylate cyclase. Indeed, a liquid to gel phase transition has been shown to occur in rat luteal cell membranes early in luteal regression (23). Such changes in the state of the plasma membrane have been shown to affect the mobility of membrane proteins (24).

The results of this study indicate that natural fatty acids of unique structure exist that may be useful agents for regulating the life span of the corpus luteum in various mammalian species.

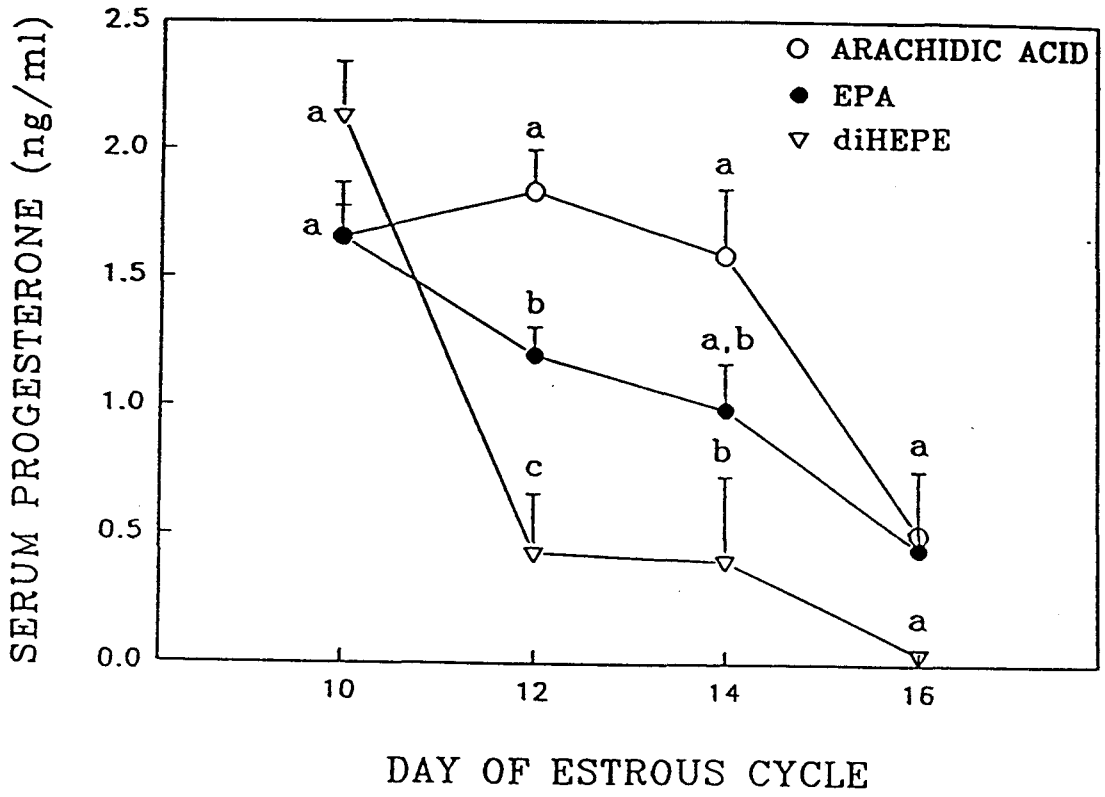


Fig. 1 Effect of methyl EPA and methyl 12,13-diHEPE (750 μg injected into the ovarian artery of ewes on day 10 of the estrous cycle) on mean (± SE) serum concentrations of progesterone compared to arachidic acid-treated controls. Mean values for the same day with different superscript letters differ ($p<0.05$).

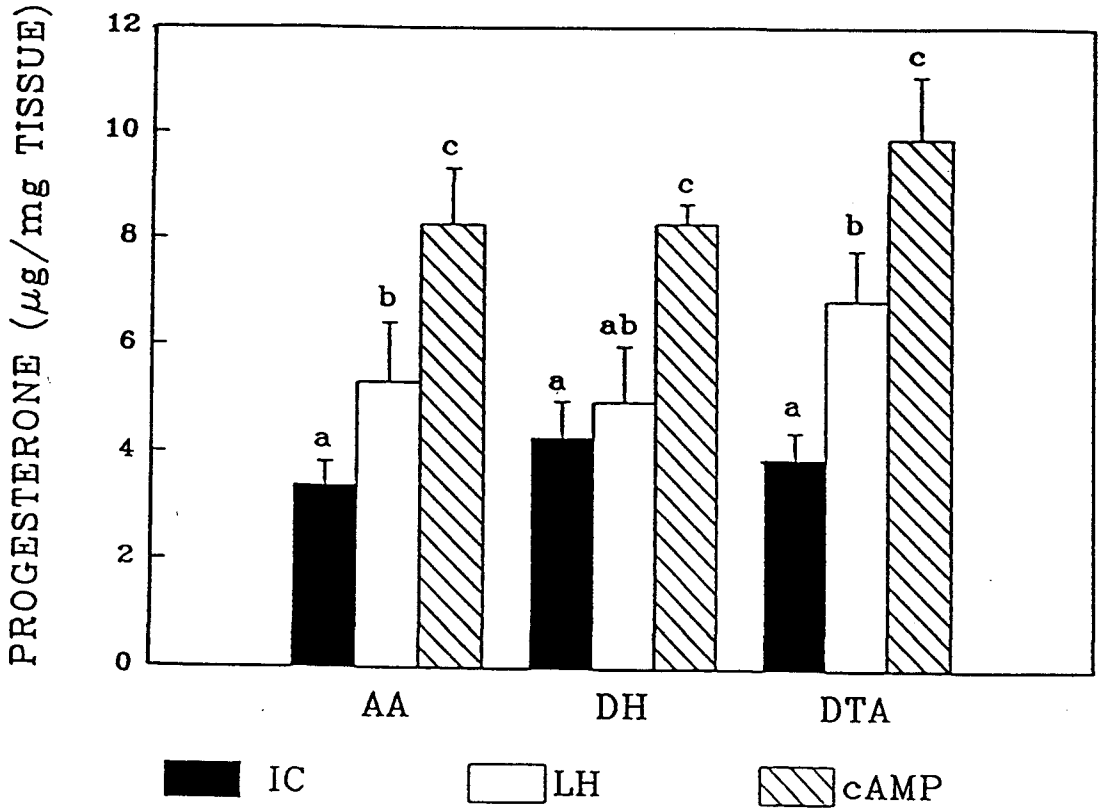


Fig. 2 Mean (± SE) progesterone production by tissues pre-treated with arachidic acid (AA), 12,13-diHEPE (DH) or docosatetraenoic acid (DTA) in response to incubation alone (IC), LH (50 ng/ml) or cAMP (20 mM). Bars with different superscript letters differ ($p<0.05$).

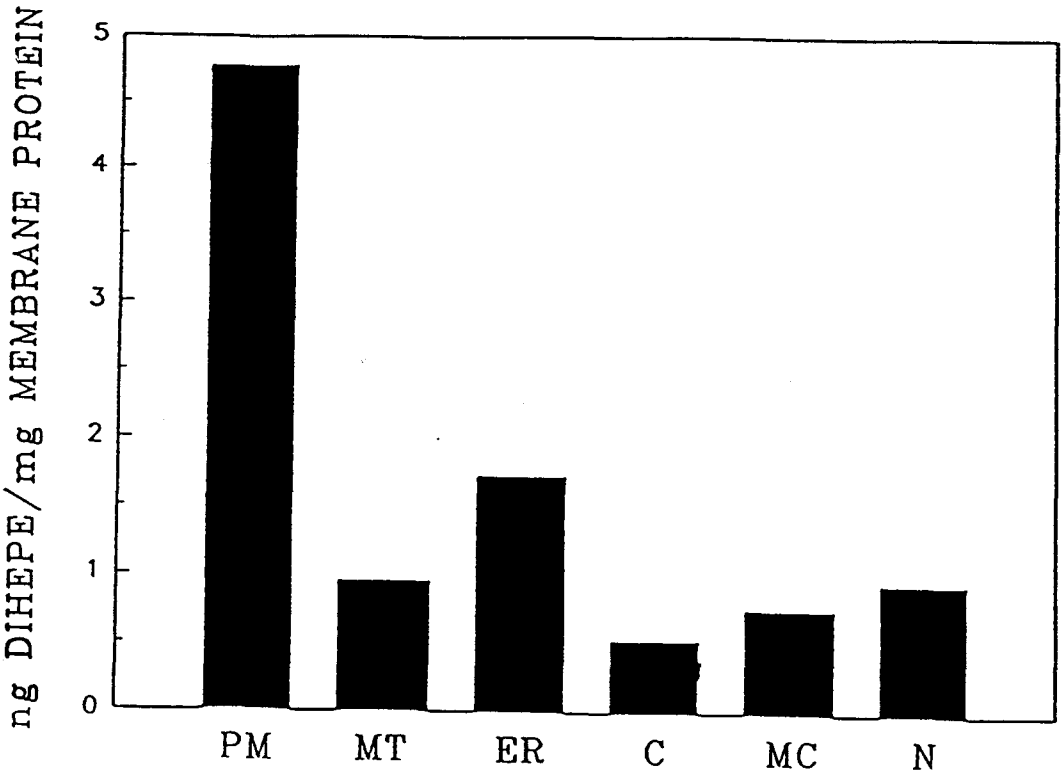


Fig. 3 Incorporation of 12,13-DiHEPE (ng/mg protein in each fraction) into the plasma membrane (PM), mitochondria (MT), endoplasmic reticulum (ER), cytoplasm (C), microsome (MC) and nuclear (N) fractions of ovine luteal tissue.

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