Judge, jury and executioner: the auto-regulation of luteal function

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Experiments were conducted to further our understanding of the cellular and molecular mechanisms that regulate luteal function in ewes. Inhibition of protein kinase A (PKA) reduced ($P<0.05$) secretion of progesterone from both small and large steroidogenic luteal cells. In addition, the relative phosphorylation state of steriodogenic acute regulatory protein (StAR) was more than twice as high ($P<0.05$) in large vs small luteal cells. Large steroidogenic luteal cells appear to contain constitutively active PKA and increased concentrations of phosphorylated StAR which play a role in the increased basal rate of secretion of progesterone.

To determine if intraluteal secretion of prostaglandin (PG) $F_2\alpha$ was required for luteolysis, ewes on day 10 of the estrous cycle received intraluteal implants of a biodegradable polymer containing 0, 1 or 10 mg of indomethacin, to prevent intraluteal synthesis of $PGF_2\alpha$. On day 18, luteal weights in ewes receiving 1 mg of indomethacin were greater ($P<0.05$) than controls and those receiving 10 mg were greater ($P<0.05$) than either of the other two groups. Concentrations of progesterone in serum were also increased ($P<0.05$) from days 13 to 16 of the estrous cycle in ewes receiving 10 mg of indomethacin. Although not required for decreased production of progesterone at the end of the cycle, intraluteal secretion of $PGF_2\alpha$ appears to be required for normal luteolysis.

To ascertain if oxytocin mediates the indirect effects of $PGF_2\alpha$ on small luteal cells, the effects of 0, 0.1, 1 or 10 mM oxytocin on intracellular concentrations of calcium were quantified. There was a dose-dependent increase ($P<0.05$) in the number of small luteal cells responding to oxytocin. Thus, oxytocin induces increased calcium levels and perhaps apoptotic cell death in small luteal cells. Concentrations of progesterone, similar to those present in corpora lutea (~ 30 μg/g), prevented the increased intracellular concentrations of calcium ($P<0.05$) stimulated by oxytocin in small cells. In large luteal cells the response to progesterone

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was variable. There was no consistent effect of high quantities of estradiol, testosterone or cortisol in either cell type. It was concluded that normal luteal concentrations of progesterone prevent the oxytocin and perhaps the $PGF_2\alpha$-induced increase in the number of small and large luteal cells which respond to these hormones with increased intracellular concentrations of calcium.

In summary, large ovine luteal cells produce high basal levels of progesterone, at least in part, due to a constituitively active form of PKA and an enhanced phosphorylation state of StAR. During luteolysis $PGF_2\alpha$ of uterine origin reduces the secretion of progesterone from the corpus luteum, but intraluteal production of $PGF_2\alpha$ is required for normal luteolysis. Binding of $PGF_2\alpha$ to receptors on large luteal cells stimulates the secretion of oxytocin which appears to activate PKC and may also inhibit steroidogenesis in small luteal cells. $PGF_2\alpha$ also activates COX-2 in large luteal cells which leads to secretion of $PGF_2\alpha$. Once intraluteal concentrations of progesterone have decreased, oxytocin binding to its receptors on small luteal cells also results in increased levels of intracellular calcium and presumably apoptosis. Increased secretion of $PGF_2\alpha$ from large luteal cells activates calcium channels which likely results in apoptotic death of this cell type.

Introduction

It has been known for decades that the parenchyma of the corpus luteum in many mammalian species includes two distinct types of steroidogenic cells (Corner 1919; Warbritton 1934; Mossman & Duke 1973), both of which secrete progesterone (Lemon & Loir 1977; Ursely & Leymarie 1979; Koos & Hansel 1981; Fitz *et al.* 1982; Rodgers & O'Shea 1982). The two types of steroidogenic luteal cells can be differentiated based on morphological and biochemical characteristics, as well as the follicular source of origin (Niswender *et al.* 1985).

Small steroidogenic luteal cells range from 12 to 22 mm in diameter (Fitz *et al.* 1982; Rodgers & O'Shea 1982). They are more numerous than large luteal cells and can be identified at the light microscopic level by their size, characteristic elongated shape, irregularly shaped nucleus and the presence of lipid droplets (O'Shea *et al.* 1979; Farin *et al.* 1986). The diameter of large steroidogenic luteal cells ranges from 22 to 35 μm with occasional cells having diameters greater than 35 μm (Donaldson & Hansel 1965; Deane *et al.* 1966; Fitz *et al.* 1982). At the light microscopic level large luteal cells usually appear spherical or polyhedral and the nucleus appears rounded. Small protein-containing secretory granules are usually detectable in the cytoplasm and large luteal cells in ewes, but not in cows, are essentially devoid of lipid droplets.

At the ultrastructural level both small and large luteal cells have numerous mitochondria and an abundance of smooth endoplasmic reticulum characteristic of other steroid-secreting cells (Christensen & Gillim 1969). However, large luteal cells also possess numerous Golgi complexes, an abundance of rough endoplasmic reticulum and numerous membrane-bound secretory granules characteristic of protein-secreting cells (Deane *et al.* 1966; Enders 1973; O'Shea *et al.* 1979). For further details regarding the morphological characteristics of the two steroidogenic luteal cells types see Rodgers & O'Shea (1982) or Niswender *et al.* (1985).

In addition to their morphological differences, small and large luteal cells have many biochemical differences in receptor content, second messenger function, and steroidogenic characteristics. Small cells have receptors for luteinizing hormone (LH) coupled to the protein kinase A (PKA) second messenger pathway which stimulates many components of the synthetic pathway for progesterone. Secretion of progesterone can be increased as much as 20-fold following stimulation of small cells with LH or analogs of cAMP (Fitz *et al.* 1982; Alila *et al.* 1988). Activation of the PKC pathway in small luteal cells inhibits LH-stimulated secretion of progesterone (Wiltbank *et al.* 1991). Large luteal cells have receptors for prostaglandin (PG) $F_2\alpha$ and PGE2 but are devoid of receptors for LH except under some conditions (Fitz *et al.* 1982; Harrison *et al.* 1987). Large cells have little, if any, response to LH in terms of increased secretion of progesterone (Hoyer & Niswender 1986). Basal secretion rates of progesterone by large luteal cells are 10- to 20-fold higher on a per cell basis than for small luteal cells (Fitz *et al.* 1982). The mechanisms responsible for this increased, but apparently unregulated, rate of progesterone secretion are not clear. Diaz *et al.* (2002) suggested that constitutive activity of the PKA pathway was likely involved since specific inhibitors of this pathway reduced secretion of progesterone by large luteal cells. Stimulation of large cells by $PGF_2\alpha$ results in activation of the PKC second messenger pathway which inhibits synthesis of progesterone by acting at multiple sites in the steroidogenic pathway (Wiltbank *et al.* 1991; Juengel & Niswender 1999). Large luteal cells also respond to $PGF_2\alpha$ with increased concentrations of intracellular calcium (Wiltbank *et al.* 1989; Wegner *et al.* 1990) which appears to be the key event in initiating the processes involved in apoptotic cell death (Vinatier *et al.* 1996).

The purpose of the research presented in the remainder of this publication was to address four specific questions regarding the cellular regulation of luteal function.

Regulation of progesterone biosynthesis in the two cell types

One of the more intriguing questions remaining to be answered regarding regulation of progesterone biosynthesis: What mechanisms are responsible for the high basal rate of secretion of progesterone from large luteal cells? To determine if PKA was tonically active in large cells resulting in continual stimulation of steroidogenesis we utilized a specific PKA inhibitor (PKI) and the adenylate cyclase activator forskolin as a positive control. The effects on progesterone and cAMP production were quantified following treatment of partially purified preparations of small and large ovine luteal cells.

Treatment of small cells with forskolin (10 μM) caused an approximately 8-fold increase in progesterone secretion ($P < 0.05$) while co-treatment with the PKI (50 μM) prevented the forskolin-induced increase (Fig. 1A). These data once again document the important role of PKA in regulating the steroidogenic pathway in small luteal cells. Concentrations of cAMP in media were not different between forskolin-treated small cells in the presence or absence of the PKI, which suggested that the effect of PKI on progesterone secretion was not due to treatment-induced toxicity. In large luteal cells forskolin treatment increased progesterone production ($P < 0.05$) and both basal secretion and forskolin-stimulated secretion of progesterone was decreased more than 5-fold ($P < 0.05$) by treatment with PKI (Fig. 1B). The modest but significant increase in progesterone production following forskolin treatment may have been due to contamination of the preparation of large luteal cells with a small number of clumps of small luteal cells. Forskolin treatment increased cAMP concentrations almost 6-fold ($P < 0.05$) in large luteal cells while progesterone production only doubled. The increase in cAMP was prevented by treatment with PKI. Thus, the effects of PKI were dramatic on reducing progesterone production by large luteal cells but the effects on cAMP concentrations were non-existent (basal) or less dramatic (forskolin treated). These data are consistent with the hypothesis that large luteal cells contain a constitutively active form of PKA

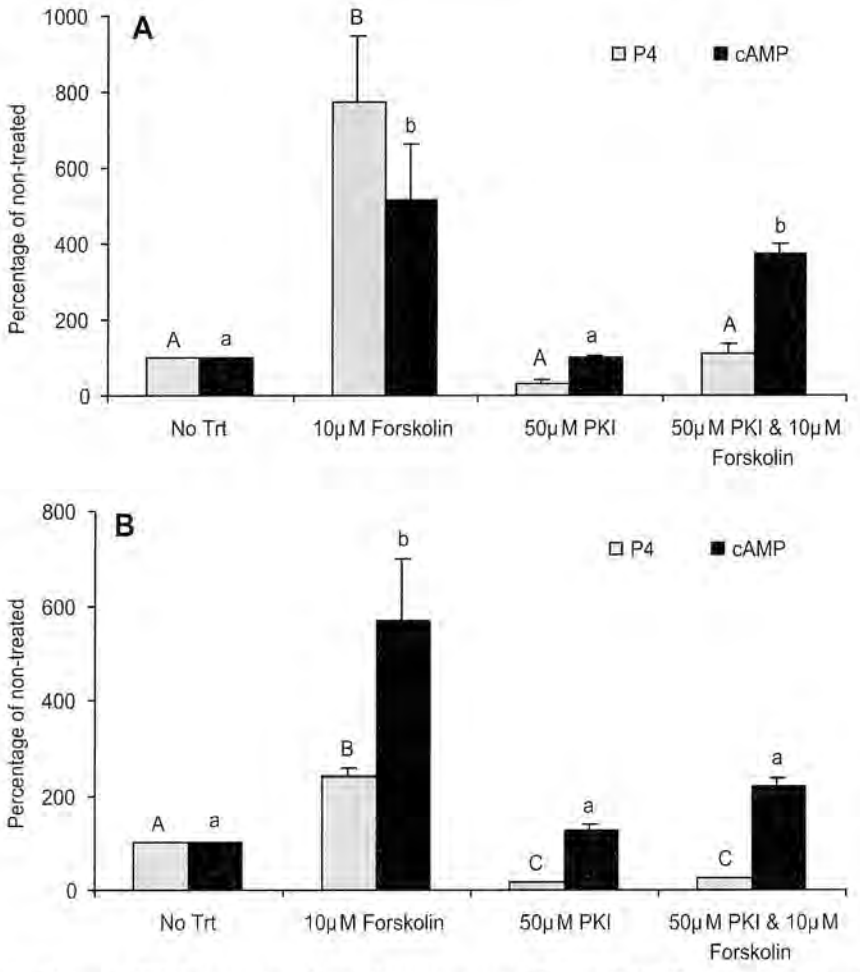

Fig. 1. Mean (± SEM) concentrations of progesterone (P4) and cAMP in media following culture of small (A) and large (B) luteal cells. Columns with different letters are different ($P < 0.05$) for mean concentrations of progesterone or cAMP.

Steroidogenic acute regulatory protein (StAR) is an important component for the transport of cholesterol to the mitochondrial membrane, the rate-limiting step in progesterone biosynthesis (Niswender 2002). Since phosphorylation is thought to be key for regulating the biological activity of StAR (Arakane *et al.* 1997) we next quantified the relative concentrations and phosphorylation state of this protein. A modified sandwich enzyme linked immunoassay (ELISA) was developed to quantify the concentrations and relative phosphorylation states of StAR in the two cell types as a biochemical marker of PKA activity (Bogan 2006). There were no effects of treatment on concentrations of StAR protein in either cell type, and no correlation between concentrations of StAR protein and progesterone in media (data not shown). However, StAR was more extensively phosphorylated under basal conditions in large compared to small luteal cells (Fig. 2; $P < 0.05$) and there was a significant decrease ($P < 0.05$) in the phosphorylation state of StAR in control or forskolin-treated large luteal cells following PKI treatment. Treatment of small luteal cells with PKI did not significantly reduce the phosphorylation state of

StAR in control cells but did reduce this parameter in forskolin-treated small cells. The relative phosphorylation state of StAR and concentrations of progesterone in media were significantly correlated when all treatments in both cell types were evaluated ($r = .71$; $P < 0.05$). Thus, the high basal rate of secretion of progesterone by large luteal cells is associated with tonically active PKA and increased phosphorylation of StAR. Degree of phosphorylation, but not changes in the quantity of StAR, appears to be a primary effect of the PKA pathway on activity of StAR protein in ovine large luteal cells.

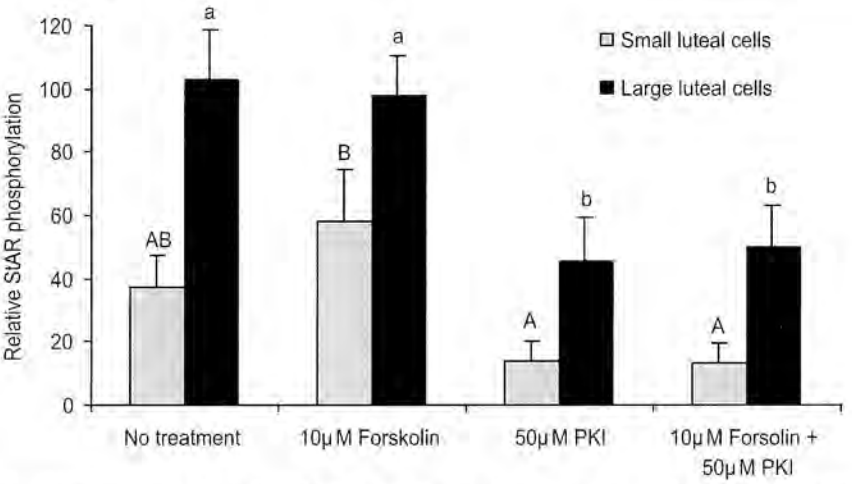

Fig. 2. Relative StAR phosphorylation state in small and large luteal cells, expressed as percentage of StAR phosphorylation compared to that in standards. Forskolin/PMA-treated mixed luteal cells were used as the 100% phosphorylated standard. Error bars indicate one SEM; columns with different letters are different ($P < 0.05$) for each cell type.

Regulation of luteolysis

The generally accepted model for regression of the corpus luteum in ruminants is that oxytocin secretion from the neuro-hypophysis or the corpus luteum stimulates the secretion of $PGF_2\alpha$ from the uterus (Bazer *et al.* 1991; Silvia *et al.* 1991). The $PGF_2\alpha$ then stimulates oxytocin secretion from large luteal cells in the corpus luteum which in turn stimulates more secretion of $PGF_2\alpha$ from the uterus establishing a positive feedback loop which ultimately causes decreased secretion of progesterone and death of the luteal cells. There are three unanswered questions regarding the cellular and molecular mechanisms involved in luteolysis that are not addressed by the existing model. First, does normal luteolysis require intraluteal as well as uterine production of $PGF_2\alpha$? Second, since small luteal cells do not have receptors for $PGF_2\alpha$, how does this hormone cause these cells to undergo apoptosis? Third, does progesterone act within the corpus luteum to influence the process of luteolysis? We have conducted experiments designed to directly address these questions.

Is intraluteal production of $PGF_2\alpha$ required for luteolysis?

Prostaglandin $F_2\alpha$ can be synthesized by corpora lutea of women (Shutt *et al.* 1976; Swanston *et al.* 1977; Patwardhan & Lanthier1980), sows (Guthrie *et al.* 1978), ewes (Rexroad & Guthrie 1979; Tsai & Wiltbank 1997), cows (Pate 1988), and rodents (Olofsson *et al.* 1992). Therefore,

we investigated whether intraluteal secretion of $PGF_2\alpha$ played a role in normal luteolysis (Griffith 2002). Ewes were administered an implant of Atrigel (100 μl) that contained 0 (n = 6), 1 (n = 8) or 10 (n = 5) mg of indomethacin to inhibit intraluteal production of $PGF_2\alpha$ into the corpus luteum on day 10 of the estrous cycle. Atrigel is a biodegradable polymer that is in a liquid state but rapidly solidifies when exposed to an aqueous environment. Indomethacin would be expected to prevent synthesis of all prostaglandins and is not specific for $PGF_2\alpha$. Samples of jugular blood were collected daily from the time of implantation until day 18 when corpora lutea were collected and weighed. Progesterone concentrations in serum were quantified by radioimmunoassay (Niswender 1973).

Although concentrations of progesterone in serum tended to be greater in ewes receiving an implant of 1 mg indomethacin compared to controls, the differences were not significant (Fig. 3). Concentrations of progesterone were greater ($P < 0.02$) from days 13-16 in ewes which received an implant containing 10 mg of indomethacin than in controls. It seems likely that there is continuous negative regulation of luteal production of progesterone by $PGF_2\alpha$ during the mid-luteal phase of the estrous cycle. By days 17-18 progesterone concentrations had reached follicular phase levels in all treatment groups indicating that the decreased secretion of progesterone associated with luteolysis had occurred. However, when luteal weights were analyzed (Fig. 4), corpora lutea which received implants containing 1 mg of indomethacin were heavier ($P < 0.05$) than controls and corpora lutea which received implants containing 10 mg indomethacin were heavier than either of the other groups with weights comparable to those observed on day 10 of the estrous cycle in similar sheep (Niswender *et al.* 1985). Thus, the normal processes responsible for reduced luteal weights did not occur in corpora lutea that contained an indomethacin implant presumably due to the lack of luteal production of $PGF_2\alpha$.

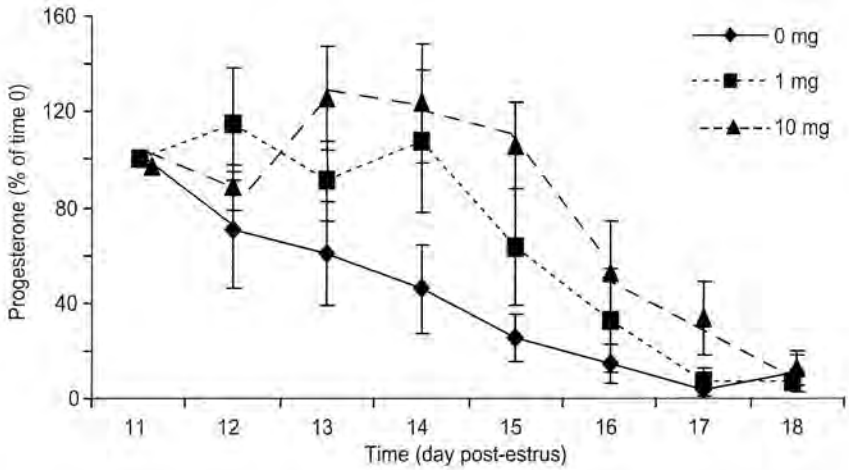

Fig. 3. Mean (± SEM) concentrations of progesterone in sera collected from ewes administered Atrigel alone (0 mg) or Atrigel containing 1 or 10 mg of indomethacin. Progesterone concentrations were greater ($P < 0.02$) on days 13-16 in ewes administered 10 mg indomethacin compared to controls.

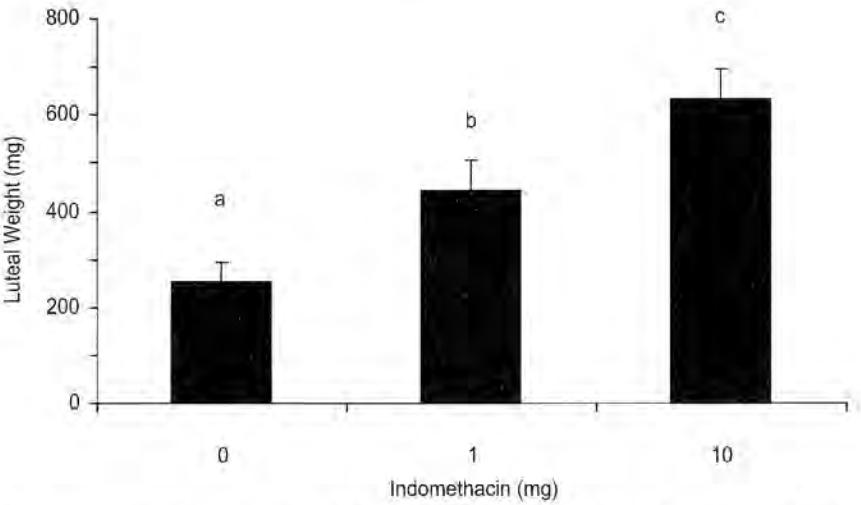

Fig. 4. Mean (± SEM) luteal weights following administration of intraluteal Atrigel implants containing 0, 1 or 10 mg indomethacin. Means with different letters were different ($P < 0.05$).

To determine if the effect of indomethacin was local, within the corpus luteum, or via a systemic mechanism an additional experiment was performed in ewes which had a single corpus luteum on each ovary. On day 11 of the estrous cycle ewes ($n = 8$) were administered an Atrigel implant in one corpus luteum and the corpus luteum on the opposite ovary received an implant containing 1 mg indomethacin. On day 18 of the estrous cycle the corpora lutea which received an implant containing 1 mg of indomethacin weighed more (412 ± 83 mg) than corpora lutea treated with Atrigel alone (275 ± 24 mg; $p < 0.05$). Thus, the effects of the intraluteal indomethacin implants appeared to be local and not systemic.

It was concluded that intraluteal production of $PGF_2\alpha$ is an important component of the normal luteolytic process. These data also support the previous suggestions that the anti-steroidogenic actions of $PGF_2\alpha$ can be separated from its luteolytic actions. McGuire *et al.* (1994) first demonstrated that intraluteal administration of PMA to activate the PKC second messenger pathway decreased serum concentrations of progesterone to follicular phase levels but had no effect on luteal weights, even though the ewes had re-ovulated. Juengel *et al.* (2000) suggested that low doses of $PGF_2\alpha$ decreased progesterone synthesis but that higher doses were required for structural regression of the corpus luteum. The current data support this suggestion and further indicate that the lower concentrations of $PGF_2\alpha$ associated with decreased progesterone production are produced by the uterus. Thus, the uterus provides the $PGF_2\alpha$ that initiates the process of luteolysis and specifically provides the signal for reduced secretion of progesterone. However, intraluteal production of $PGF_2\alpha$ is required for loss of luteal weight and cell death.

It is likely that attenuation of intraluteal secretion of $PGF_2\alpha$ may play an essential role in maintenance of the corpus luteum of pregnancy. Silva *et al.* (2000) clearly demonstrated that luteal mRNA encoding 15-hydroxyprostaglandin dehydrogenase (PGDH) and the activity of this enzyme, a cytosolic enzyme responsible for catabolism of $PGF_2\alpha$, is increased in ovine corpora lutea during the time of maternal recognition of pregnancy. The signal for increasing mRNA for PDGH activity in the corpus luteum may be interferon-tau since Ott *et al.* (personal

communication) have suggested that this hormone has direct effects at the level of the corpus luteum.

Does oxytocin control intracellular calcium concentrations in small luteal cells?

Since small luteal cells do not have receptors for $PGF_2\alpha$ (Fitz *et al.* 1982) the luteolytic effects of this hormone on this cell type have to be indirect. Oxytocin inhibited the LH-stimulated secretion of progesterone from small luteal cells which suggests that small luteal cells have receptors for oxytocin (Mayan & Niswender, unpublished data). Since large luteal cells release oxytocin when stimulated by $PGF_2\alpha$, this hormone was a likely candidate to mediate the effects of $PGF_2\alpha$ on small luteal cells. A series of experiments was performed to ascertain if oxytocin treatment of small luteal cells influenced intracellular concentrations of calcium. Increases in intracellular calcium can perturb cell function by activating endonucleases which cause DNA fragmentation (Vinatier *et al.* 1996), which occurs during regression of bovine (Juengel *et al.* 1993) and ovine luteal tissue (McGuire *et al.* 1994; Rueda *et al.* 1995; Juengel *et al.* 2000). Further, increases in intracellular calcium concentrations also induce nitric oxide production (Yi *et al.* 2004), which has also been implicated as a mediator of luteal regression (Jaroszewski & Hansel 2000; Korzekwa *et al.* 2006).

Corpora lutea were collected from 1-3 ewes for each replicate (n = 8) on days 9 or 10 of the estrous cycle, dissociated into single cell suspensions and partially purified into preparations of small and large steroidogenic luteal cells by centrifugal elutriation. Small luteal cells (50,000 per plate) were plated overnight on Matrigel-coated (BD Biosciences) 35 mm glass bottom dishes (MatTek) in DMEM containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Media were removed and cells were washed twice with fluorescence buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.5 mM $MgCl_2$ $6H_2O$, 1 mM $CaCl_2$ $2H_2O$, 10 mM HEPES, and 5 mM glucose). Cells were loaded with 3 μM Fura-2, acetoxymethlyester (Fura-2, AM) containing 0.1% Pluronic F-127 (Molecular Probes) for 30 minutes. Cells were then washed in fluorescence buffer and allowed to recover for 30 minutes before a final wash. Calcium responses were measured using an InCyt2 imaging system (Intracellular Imaging Inc.) as previously described (Shlykov & Sanborn 2004). Changes in intracellular concentrations of calcium in individual cells were measured at 340 nm and 380 nm excitation and 510 nm emission wave lengths. After measuring basal intracellular calcium concentrations for 30s, cells were stimulated with 0.1 μM- 10 μM oxytocin (Sigma Chemical Co., St. Louis, MO) and intracellular calcium concentrations measured for 5 minutes. The oxytocin receptor in ovine uterus has a Kd of approximately 10 nM (Dunlap & Stormshak 2004) so addition of 100 nM to 10 μM allowed rapid occupancy of receptors. It also is likely that intraluteal levels of oxytocin become quite high since large luteal cells produce this hormone. Data collected included percentage of cells responding to oxytocin with increased concentrations of intracellular calcium as well as the average peak height.

There was a dose-dependent increase in both the percentage of cells exhibiting increased calcium concentrations and peak height of intracellular concentrations of calcium in cells that responded following treatment with increasing quantities of oxytocin (Fig. 5). It seems likely that the increased intracellular concentrations of calcium initiate the mechanisms leading to apoptosis in this cell type. Oxytocin had no effect on intracellular concentrations of calcium in large luteal cells when studied under the same conditions as those used for small luteal cells.

Does progesterone influence luteolysis?

It is generally accepted that the corpus luteum undergoes luteolysis by apoptotic mechanisms. Apoptotic processes have been well characterized. Chromosomal condensation along the

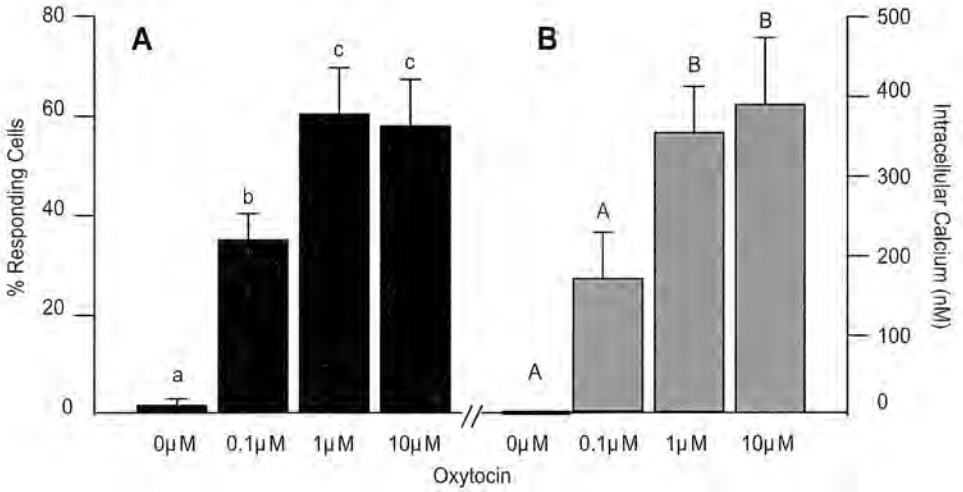

Fig. 5. Effect of various doses of oxytocin to increase intracellular calcium in individual small luteal cells. Percent of cells responsive to oxytocin is shown in panel A while the peak height of intracellular calcium is shown in panel B. Doses of oxytocin with different lower case superscripts are statistically different ($P < 0.05$). Data are from 8 replicates for percent responsive cells and 4 replicates for maximum intracellular calcium concentrations.

periphery of the nucleus and shrinkage of the cell body are early signs of apoptosis. Later the nucleus and the cytoplasm fragment forming apoptotic bodies which are phagocytosed by macrophages (Lodish *et al.* 2000). Another distinguishing characteristic of apoptosis is the formation of 185 bp fragments of genomic DNA (oligonucleosomes) as a result of activation of calcium-dependent endonucleases (Arends *et al.* 1990). Oligonucleosome formation has been used as an indicator of apoptosis in ovine (McGuire *et al.* 1994; Rueda *et al.* 1995; Juengel *et al.* 2000) and bovine (Juengel *et al.* 1993) corpora lutea following treatment with $PGF_2\alpha$.

Progesterone appears to inhibit apoptosis in a number of reproductive tissues in a variety of species. In bovine luteal cells, treatments that reduced progesterone secretion increased oligonucleosome formation (Rueda *et al.* 2000) while treatment with cAMP analogs increased progesterone production and attenuated apoptosis (Tatsukawa *et al.* 2006). Progesterone rescues luteal structure and function in the rat (Goyeneche *et al.*, 2003) by inhibiting DNA fragmentation and apoptosis. Friedman *et al.* (2000) demonstrated that progesterone protected luteal endothelial cells from TNFα-induced apoptosis. In the endometrium, decreased progesterone concentration is followed by apoptosis in epithelial cells (Rotello *et al.* 1992). Pecci *et al.* (1997) demonstrated that addition of anti-progestins increased apoptosis in rat endometrial cell lines. Thus, there is considerable evidence that progesterone may prevent apoptosis in the corpus luteum and the endometrium.

Since intracellular concentrations of calcium are thought to be a primary regulator of endonuclease activity and apoptosis, the next series of experiments was performed to evaluate the role of progesterone on the ability of oxytocin and $PGF_2\alpha$ to increase intracellular concentrations of calcium in small and large luteal cells, respectively. The first step in this study was to determine what "physiological" concentrations of progesterone were within the corpus luteum. We collected blood samples from the ovarian veins of nine ewes on day 10 of the estrous cycle and serum concentrations of progesterone were 0.9 ± 0.1 μg/ml. We also collected six blood samples following puncture of the surface of the corpus luteum with a needle and serum con-

centrations of progesterone were 0.9 ± 0.3 μg/ml. It was clear that intraluteal concentrations of progesterone were at least 1 μg/ml and likely much higher than that in the extracellular fluid surrounding the luteal cells which produce this hormone. Luteal content of progesterone during the mid-luteal phase ranges from 10-30 μg/g tissue (Silvia, 1985) and there is no evidence for storage of this hormone. Therefore, concentrations 1 to 30 μg/ml of progesterone were used for the following experiments.

In the first series of experiments 50,000 small luteal cells per plate were incubated overnight with media containing 0.1% DMSO or 1, 10 or 30 μg of progesterone, estradiol, testosterone or cortisol per ml. Steroids were dissolved in DMSO and 0.1% was added in all treatments. Intracellular concentrations of calcium were quantified as described above after addition of 10 μM oxytocin.

Both 1 and 10 μg/ml progesterone tended to decrease the number of small luteal cells responding to oxytocin while 30 μg/ml essentially abolished the response to oxytocin (Fig. 6). The effect appeared to be specific for progesterone since similar high quantities of estradiol, testosterone or cortisol did not have a consistent inhibitory effect.

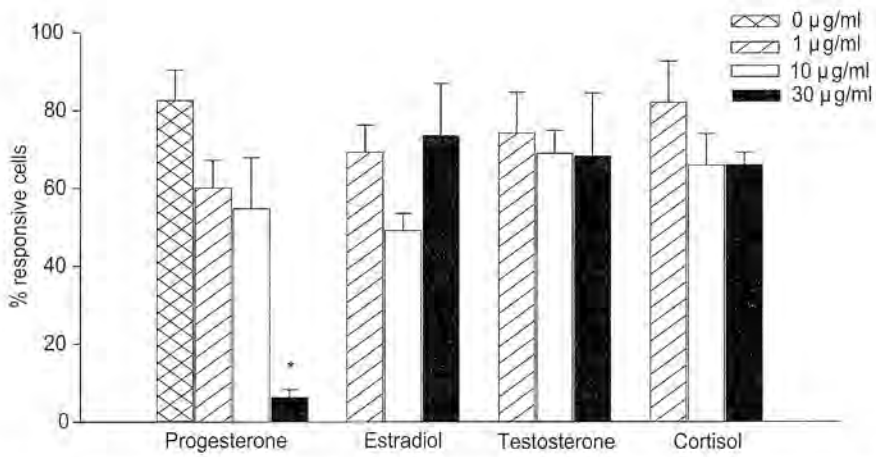

Fig. 6. Effect of steroid hormones (0, 1, 10 or 30 μg/ml) on the percent of cells responding with an increase in intracellular concentrations of calcium following oxytocin treatment (10 μM) in individual small luteal cells. Thirty μg/ml progesterone suppressed the response to oxytocin ($P < 0.0001$). Data are from 3 replicates.

The suppressive effect of progesterone on the ability of oxytocin to increase intracellular calcium concentrations is likely a direct, non-genomic action of progesterone on the oxytocin receptor. Recently it has been reported that progesterone competes with oxytocin for oxytocin receptors in the uterine endometrial plasma membrane (Bogacki *et al.* 2002; Dunlap & Stormshak 2004; Duras *et al.* 2005; Bishop & Stormshak 2006) and in CHO cells transfected with oxytocin receptor (Grazzini *et al.* 1998). Binding of oxytocin to its receptor in endometrial cells stimulates hydrolysis of phospholipase C to phosphoinositides (Mirando *et al.* 1990). Oxytocin stimulated IP3 production is attenuated in ovine uterine explants exposed to progesterone (Bishop & Stormshak 2006). Progesterone is able to suppress oxytocin-stimulated $PGF_2\alpha$ secretion from both ovine (Bishop & Stormshak 2006) and bovine endometrium (Duras *et al.* 2005). Burger *et al.* (1999) reported that large concentrations of progesterone similar to those used in the present studies abolished calcium signaling stimulated by ligand activated G-protein coupled receptors (GPCR). In contrast to the suggestion that progesterone directly interacts with the oxytocin receptor, these

authors suggest that microgram concentrations of progesterone alter the cholesterol content of caveolae where many GPCR reside and regulate their ability to increase calcium concentrations. Progesterone also attenuates oxytocin-induced increases in concentrations of intracellular calcium in bovine endometrial cells (Bogacki *et al.* 2002). Burger *et al.* (1999) suggested that progesterone may act at plasma membrane calcium channels or the membrane of the endoplasmic reticulum to alter calcium signaling. The recent publication by Ashley *et al.* (2006) who identified a membrane receptor for progesterone in sheep corpora lutea, that appears to be localized to the membranes of the endoplasmic reticulum, makes the role of progesterone in luteal function even more confusing.

In large luteal cells the ability of progesterone to inhibit $PGF_2\alpha$ induced increases in intraluteal concentrations of calcium was not so apparent (Fig. 7). There was no significant decrease in the number of cells responding to $PGF_2\alpha$ compared to controls. However, in six replicates of this experiment it was clear that the response was highly variable. In three replicates there was no response to progesterone while in the other three progesterone at 30 μg/ml reduced ($P<0.05$) the number of large luteal cells which responded to $PGF_2\alpha$ treatment by over 50% (Fig. 8).

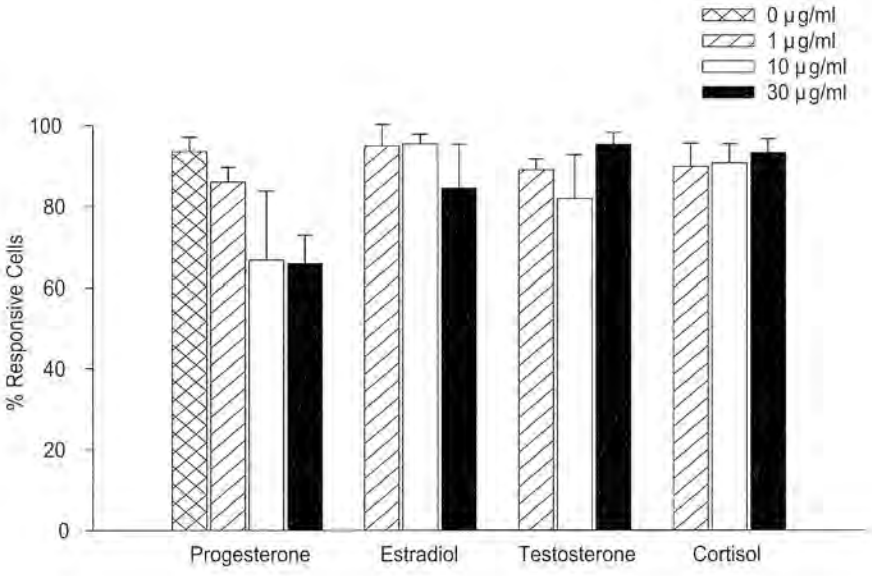

Fig. 7. Effect of steroid hormones (0, 1, 10 or 30 μg/ml) on the number of large luteal cells responding with increased intracellular concentrations of calcium following $PGF_2\alpha$ treatment (1 μM). There was no significant effect of any steroid hormone. Data are from 6 replicates.

It may not be surprising that large luteal cells were not as responsive to progesterone as small luteal cells. Large cells secrete high levels of progesterone at all times and thus, physiological levels at the cell membrane may always be quite high. In fact, it would not be surprising if the levels of progesterone in extracellular fluid surrounding large luteal cells was much higher than the maximum dose (30 μg/ml) used for these studies. We attempted to address this issue in two ways. First, we removed the media from large cells to reduce progesterone content and then incubated them for only 4 hr in the presence of the differing levels of progesterone. Results following these procedures were not consistent. We also attempted to use RU486, a

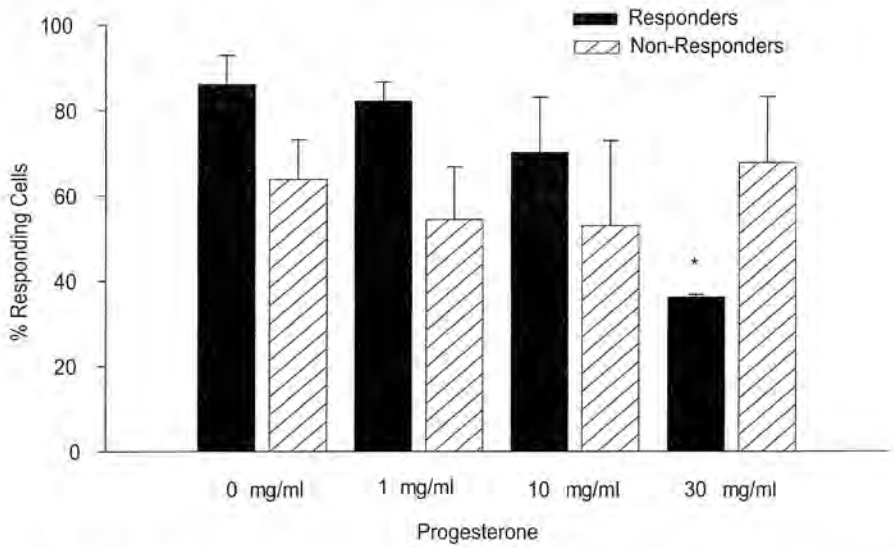

Fig. 8. Effects of 1 μM $PGF_2\alpha$ on the percent of large cells responding with an increase in intracellular calcium concentrations in the presence of various concentrations of progesterone. Data are shown as replicates in which cells responded (n = 3) or did not respond (n = 3) to progesterone (30 μg/ml) treatment. *Percent responding cells with 30 μg/ml progesterone is statistically different ($P < 0.05$) than % responding with 0 μg/ml progesterone.

known inhibitor of progesterone's genomic actions but this analog had no effect. This was not surprising since the actions of progesterone on the response to oxytocin and $PGF_2\alpha$ are likely mediated by a membrane receptor for this steroid hormone. It would seem to be unusual that any steroid hormone receptor would require such high concentrations of ligand to act through any currently accepted model. However, the data described above clearly suggest that progesterone secretion and therefore concentrations within the corpus luteum must decrease before oxytocin and likely $PGF_2\alpha$ can induce the increases in intracellular calcium concentrations that are associated with apoptotic cell death.

It is clear that cytokines and the immune system also play a role in the structural demise of the corpus luteum (Pate & Keyes 2001; Davis & Rueda 2002). However, it was outside the scope of this review to address the ever increasing information regarding the role of the immune system in regulating the functions of the corpus luteum.

Conclusions

Our current model of the mechanisms controlling luteolysis in ewes (Fig. 9) is that pulses of $PGF_2\alpha$ of uterine origin activate PKC in steroidogenic luteal cells (Wiltbank *et al.* 1991) which reduces the activity of many of the components of the progesterone synthetic pathway (Juengel & Niswender 1999). $PGF_2\alpha$ also induces secretion of oxytocin from large luteal cells which, once intra-luteal levels of progesterone are reduced, allows increased concentrations of calcium and ultimately death of small luteal cells. This is supported by the findings of Braden *et al.* (1988) who demonstrated that numbers of small luteal cell decreased sooner than numbers of large luteal cells following treatment with $PGF_2\alpha$ in ewes. Uterine $PGF_2\alpha$a also increases the activity of COX-2 and synthesis of $PGF_2\alpha$ from large luteal cells. Once the secretion of progesterone has been decreased, $PGF_2\alpha$ also acts to increase intracellular concentrations of calcium in large luteal cells and induces apoptotic death of this cell type.

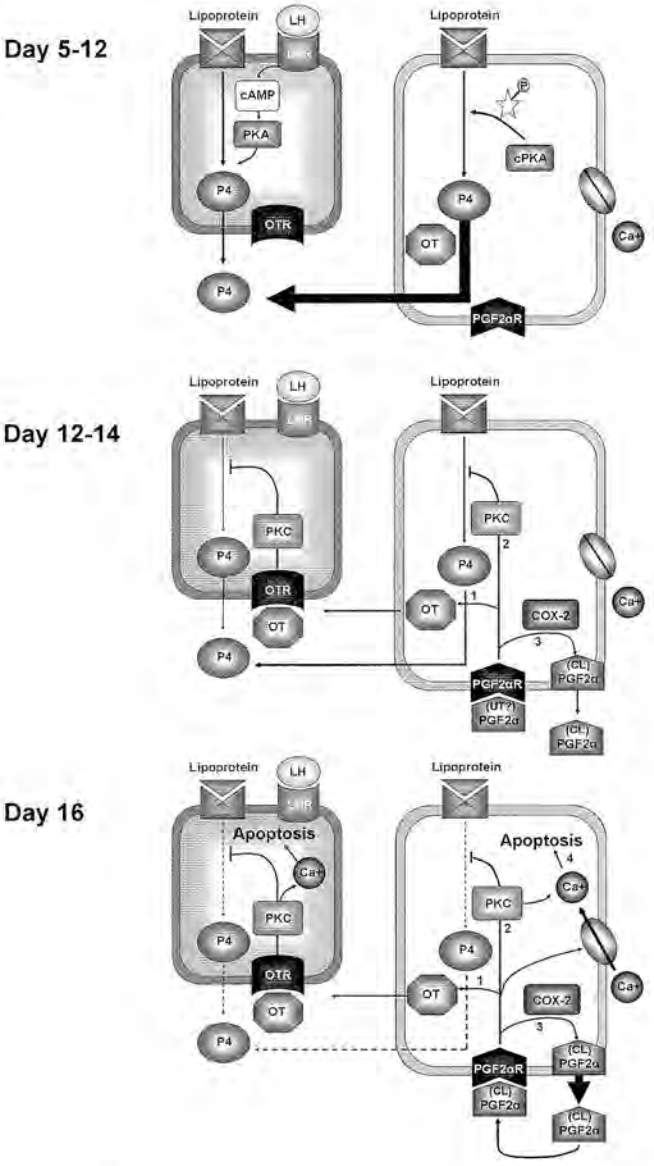

Fig. 9. Current hypothetical model of the intraluteal mechanisms involved in regulation of luteal function in ruminants. During the mid-luteal phase of the estrous cycle (days 5-12) large luteal cells produce the majority of the progesterone, at least in part, due to constitutively active PKA (cPKA) and the increased phosphorylation of steroidogenic acute regulatory protein (StAR). Small luteal cells respond to LH with increased secretion of progesterone. During the early luteolytic phase (days 12-14) large luteal cells respond to small doses of $PGF_2\alpha$ from the uterus with: 1) secretion of oxytocin; 2) activation of PKC which reduces steroidogenesis; and 3) increased activity of cycloxygenase (COX)-2. During this time the high intraluteal levels of progesterone likely prevent the actions of oxytocin on small luteal cells and the ability of $PGF_2\alpha$ to increase calcium in large luteal cells. During the late luteolytic phase of the estrous cycle (day 16), when progesterone secretion has diminished by 80%, oxytocin binds to its receptor in small luteal cells reducing further the secretion of progesterone and increasing intracellular levels of calcium and apoptosis. In large luteal cells secretion of $PGF_2\alpha$ is further increased and this hormone continues to stimulate oxytocin release, activate PKC, and stimulate COX-2 activity. However, the high levels of $PGF_2\alpha$ secreted by large luteal cells also induces increases in intracellular calcium which likely leads to apoptosis and death of this cell type.

We propose that the previous models for luteolysis are incomplete. The data presented herein strongly suggest that the decision regarding ultimate fate of luteal cells is orchestrated by the corpus luteum itself. The initiation of the events is controlled by the uterus but an intricate system of checks and balances allows the corpus luteum to control its own destiny. The large luteal cells preside over this activity responding initially to $PGF_2\alpha$ with an attenuation of progesterone production and an increase in secretion of oxytocin. Together the small and large luteal cells, now no longer protected by production of progesterone, can respond to oxytocin or $PGF_2\alpha$ with increased intracellular concentrations of calcium and apoptosis. During early pregnancy, $PGF_2\alpha$ production by large luteal cell is decreased due to the increased activity of PGDH which is likely important for pregnancy to proceed. Thus, the corpus luteum plays the role of being its own judge, jury, and executioner.

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