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Investigations into bovine luteolytic mechanisms utilizing a
long-term cell culture system

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University of New Hampshire, 1988

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INVESTIGATIONS INTO BOVINE LUTEOLYTIC MECHANISMS UTILIZING A LONG-TERM CELL CULTURE SYSTEM

by

Shari Jean Litch
B.S., Tufts University, 1982
M.S., University of New Hampshire, 1985

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
The requirements for the Degree of

Doctor of Philosophy
in
Animal and Nutritional Sciences

May, 1988

This thesis has been examined and approved.

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ACKNOWLEDGEMENTS

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ABSTRACT

INVESTIGATIONS INTO BOVINE LUTEOLYTIC MECHANISMS
UTILIZING A LONG-TERM CELL CULTURE SYSTEM

by

Shari J. Litch
University of New Hampshire, May, 1988

Luteal regression in the cow occurs every cycle in which pregnancy does not occur. The agent which induces luteolysis is a uterine prostaglandin, $PGF_{2\alpha}$, although the mechanism of its action remains unknown. The purpose of this study was to investigate the mode of action of $PGF_{2\alpha}$ in a long-term, serum-free culture system of bovine luteal cells. Experiment I investigated the influence of LH and $PGF_{2\alpha}$ on 3β-HSD presence in cultured luteal cells. Total numbers of cells dropped slightly throughout the 8 day culture. Numbers of 3β-positive cells also dropped in all treatment groups. 3β-HSD was best maintained in the presence of LH. $PGF_{2\alpha}$ treatment had no influence on numbers of 3β-positive cells. Experiment II investigated the role of Ca^{++} and calmodulin in the regulation of P_4 steroidogenesis. The presence of extracellular Ca^{++} is mandatory for LH stimulation of P_4 as is seen in EGTA treated medium. Ca^{++}-enriched medium (A23187) increased LH-stimulated P_4 production. P_4 in the $PGF_{2\alpha}$ treatment was not altered in either the presence or absence of elevated Ca^{++}. The production of endogenous $PGF_{2\alpha}$ was not influenced by Ca^{++} environment. Ca^{++} antagonists TMB-8 and

CCCP had no effects on basal P_4 production but did inhibit LH-stimulated P_4. Calmodulin antagonists, TFP and W-7, were only able to slightly inhibit LH-stimulated P_4 and had no effect on basal P_4. Experiment III investigated the influence of E_2 and phenol red, a weak E_2, on luteal P_4 steroidogenesis. E_2 was shown to suppress LH-stimulated P_4 in phenol-containing medium but had no effect on basal or $PGF_{2\alpha}$-induced P_4. The use of phenol-free medium resulted in higher absolute P_4 levels for control and LH treatments. E_2 suppressed LH-stimulated P_4 early in the culture but was unable to cause an effect on day 8 and 10. No statistically significant cooperative effects of E_2 + $PGF_{2\alpha}$ were found but trends suggest lowered P_4 in the presence of both of these luteolytic agents. In conclusion, the presence of low levels of LH is suggested to maintain the number of steroidogenically active cells. $PGF_{2\alpha}$ does not mediate its action through a reduction in 3β-HSD. LH-stimulated P_4 production requires free, intracellular and extracellular Ca^{++} whereas, the role of Ca^{++} in $PGF_{2\alpha}$ action remains unknown. Also, free Ca^{++} appears to be a more important regulator of luteal steroidogenesis than calmodulin-bound Ca^{++}. Estrogen appears to have a direct effect on LH-stimulated P_4 production. Phenol red appears to influence basal and LH-stimulated P_4 production. The complete mechanism of $PGF_{2\alpha}$ action remains to be elucidated.

LITERATURE REVIEW

The bovine estrous cycle is governed to a large extent by the corpus luteum (CL), a transient endocrine organ located in the ovary. The corpus luteum, or "yellow body", was so named by Marcello Malpighi in 1697 because of its yellow color in the cow. The bovine cycle has a finite length of approximately 21 days, 16 of which are dominated by the CL and the progesterone (P_4) secreted by it (Hansel et al., 1973). The CL is formed from the granulosa and thecal cell layers of an ovulated follicle. The primary function of the CL is to manufacture P_4, and thereby maintain pregnancy. If pregnancy does not occur, the CL is terminated at approximately day 19 and undergoes functional and morphological luteolysis, characterized initially by a dramatic drop in circulating plasma P_4 levels. Removal of the luteal influence allows a new batch of primary follicles to start the maturation process and just prior to ovulation, a new estrous cycle begins.

OVULATION

At the level of the hypothalamus, estradiol (E_2) from the developing follicles positively feeds back to cause the synthesis of gonadotropin-releasing hormone (GnRH) which stimulates the pulsatile release of gonadotropins from the anterior pituitary in sheep (Clarke and Cummins, 1982), rats (Levine and Ramirez, 1982), non-human primates (Knobil, 1987) and humans (Marshall and Kelch, 1986). Each cycle, several oocytes or primary follicles are stimulated to mature beyond their arrested position at prophase of the first meiotic division. The follicles are

first exposed to the glycoprotein follicle stimulating hormone (FSH), which, as its name implies, stimulates the primary ooycte from its resting state to continue through the meiotic divisions (Hafez, 1980). The maturation continues with the formation of granulosa and thecal cell layers and at the stage of the tertiary follicle these layers begin synthesizing estrogens, presumably by the proposed mechanism of the "two cell theory" (Falck, 1959). LH bound to receptors on the thecal layer initiates the conversion of cholesterol to pregnenolone (P_5). The granulosa cells, unable to complete the pathway of P_5 to testosterone, must transport this sub-strate across the basement membrane to the theca. Stores of P_5 in the theca are converted to androstenedione and transported back to the granulosa cells for the production of testosterone. Under the influence to FSH, testosterone is aromatized to 17β-estradiol (E_2) in the granulosa cell layer only and shuttled back across the basement membrane to the blood stream.

Exposure to E_2 and to FSH signals the granulosa cells to form receptors for luteinizing hormone (LH), which is also a glycoprotein released from the anterior pituitary. LH and FSH are very similar in structure, both being composed of identical alpha subunits and slightly dif-ferent beta subunits. The release of LH is stimulated via positive feedback of estradiol pro-duced by the growing follicle. Estradiol from the ovary is secreted and carried via the systemic circulation to the hypothalamus where it stimulates the release of luteinizing hormone- releas-ing hormone (LH-RH). LH-RH is transported down neurosecretory neurons to the hypothalamo-hypophyseal portal veins and to its site of action in the anterior pituitary. The resulting product is LH which is produced in secretory cells and released into the bloodstream. LH binds to plasma membrane receptors on the granulosa cell of the follicle and initiates an increase in cyclic adenosine monophosphate (cAMP) which results in elevated levels of P_4

(Kolena and Channing, 1972). These steroid interactions cause the fibroblasts to activate collagenase and an inflammatory-type reaction in a selected mature follicle. Increased blood flow, connective tissue degradation and follicular contractions cause the follicle to rupture and the ovum is released at the apex. All remaining tertiary follicles will undergo atresia, as normally in the cow only one egg is ovulated per cycle (Hafez, 1980). At this time the collapsing granulosa and thecal cell layers of the follicle undergo morphological luteinization and switch from production of primarily estradiol to progesterone.

LUTEINIZATION

Just prior to ovulation, the cow spends approximately ten hours in estrus. At this time, if the animal is inseminated, pregnancy can result. Whether or not pregnancy occurs, the young CL begins to become morphologically and functionally active.

The quantity of luteal cells is determined at or around ovulation and this number does not change throughout the luteal phase in the rat (Meyer, 1980). The same study suggested that increases in CL weight with age are mainly due to hypertrophy. The rat CL appears richly vascularized as early as the first day of pregnancy (Meyers, 1980). In the human, thin walled vessels invade the granulosa from the thecal layer very shortly after ovulation and begin to establish the capillary network (Balboni, 1983). Vascular intervention proceeds and it appears in the cow that vessels leading from the uterus to the ovary with the CL are significantly more developed and have larger diameters than those to the contralateral ovary (Ginther and Del Campo, 1974 and Ford and Chenault, 1981). The mature CL is characterized by densely-staining granules, profuse smooth endoplasmic reticulum and numerous lipid droplets (Gem-

mell and Stacy, 1979).

Two cell populations, large and small, have been identified in bovine corpora lutea (Gier and Marion, 1961). In later studies, cells <20 μm were designated small and found to respond to LH while those >20 μm were designated large and considered responsible for basal P_4 production (Ursely and Lemarie, 1979; Koos and Hansel, 1981). Morphologically, small luteal cells (15-18 μm) were shown to have low cytoplasmic/nuclear ratio and contain some mitochondria, lipid droplets, dense granules, lysosomes, and endoplasmic reticulum. Large luteal cells (18-45 μm) were shown to have high cytoplasmic/nuclear ratio and contain numerous mitochondria, lipid droplets, dense granules, and lysosomes (Chegini et al., 1984). Fitz et al. (1982) found two cell populations in ovine corpora lutea, the smaller cells (12-22 μm in diameter) which also had significantly higher numbers of LH/hCG receptors than the large cells (23-25 μm). The large cells were found to have more prostaglandin and estrogen receptors than the small cells (Fitz et al., 1982; Glass et al., 1984).

Alila and Hansel (1984) used monc ·lonal antibodies raised against granulosa and thecal cell surface antigens to determine the follicular origin of large and small bovine luteal cells. They concluded that initially large cells are derived from the granulosa layer and small cells from the thecal cells. As the CL matures, some of the small cells of thecal origin develop into large cells and some granulosa-derived large cells die. Therefore, at the end of the cycle the luteal cell population should include small cells of thecal origin, and a mixed population of granulosa-derived and thecal-derived large luteal cells. Ovine luteal populations have been shown, using the enzyme 3β-hydroxysteroid dehydrogenase as a steroidogeneic marker, to increase in both size and number early in the cycle but undergo a preferential loss of small

luteal cells around the time of luteolysis (Schwall et al., 1986).

LH receptors are found on both cell types but only the small cells respond to LH-stimulation while the large cells are primarily responsible for basal P_4 production (Rodger and O'Shea, 1982;Harrison et al., 1987). A French research group has recently shown the presence of $PGF_{2\alpha}$ receptors on small bovine luteal cells and have demonstrated decreased P_4 in response to high levels of exogenous $PGF_{2\alpha}$ (Benhaim et al., 1987). As the CL develops, its ability to bind and respond to LH or hCG decreases, and its sensitivity to $PGF_{2\alpha}$ increases (Rao et al., 1983).

LUTEOTROPISM

Luteotropism refers to the functional capacity of luteal cells to synthesize P_4. This production of steroid is usually driven by a hormone(s) of pituitary origin, denoted a luteotropic agent or complex. The main luteotropin varies from species to species, but in the cow, Donaldson and Hansel (1965) found LH to be quite effective in extending the functional capacity of the CL beyond the normal length of the estrous cycle. Human chorionic gonadotropin (hCG) also appeared to have luteotropic properties and extended the estrous cycle in the cow (Wiltbank et al., 1961 and Seguin et al., 1977). Receptors for LH, which also bind hCG, are known to exist on the plasma membrane of luteal cells (Rao, 1979) and, once bound to the receptor, these seem to interact with an adenylate cyclase complex via a guanine nucleotide binding protein (G-protein) within the membrane. G-proteins are a family of signal transducers, each comprised of 3 subunits, α, β, and γ, usually located on the cytosolic side of the membrane. The function of the α-subunit is to act as a GTPase while the β/γ complex acts to

anchor the protein in the membrane (Sternweis, 1986). The G-protein, specifically G_s in the case of adenylate cyclase activation, interacts with the hormone-receptor complex, hydrolyzes GTP to GMP and couples the receptor complex to the enzyme (review, Spiegel, 1987).

Adenylate Cyclase

The inactive form of adenylate cyclase is composed of a regulatory and catalytic unit. When the LH-receptor complex is translocated through the membrane and coupled with the n_β-subunit of the regulatory unit of adenylate cyclase, it becomes activated and a series of events is initiated. Initially the induction of LH-stimulation is dependent on magnesium (Mg^{++}) binding to the n_β- subunit (Birnbaumer and Kirchick, 1983) and the separation of the n_α and n_β-subunits. Also, GTP binding to the n_α-subunit of the regulatory unit is mandatory for activation (Dufau et al., 1982). Once these prerequisites are accomplished, the catalytic unit couples with the n_α-subunit of the regulatory unit and $Mg^{++}ATP$ is converted to cAMP. This step is crucial in that sufficient levels of cAMP are necessary to trigger a series of cytoplasmic events necessary for the synthesis of P_4. LH-stimulated accumulation of cAMP activates a protein kinase (PK-A) (Ling and Marsh, 1977) which stimulates cholesterol ester hydrolase to increase free cholesterol and acts on ribosomes to release a labile protein (Hermier et al., 1971). Free cholesterol is then able to diffuse through the mitochondrial membrane and enter the cholesterol-side chain cleavage pathway. Two oxidation steps bring cholesterol to 20,22-di-OH-cholesterol. The labile protein may cause cytoplasmic polyphosphoinositide (PIP) to enter the mitochondria and make 20,22-di-OH- cholesterol more available to the cytochrome P_{450} complex located on the inner mitochondrial membrane (Farese, 1983). At this time oxi-

dized cytochrome P_{450} binds to cholesterol and becomes reduced. In the presence of oxygen, reduced P_{450}- cholesterol reacts to form pregnenolone (P_5) and oxidized P_{450}. This oxidized P_{450} may again be coupled with additional 20,22-di-OH-cholesterol. The final step involves transfer of pregnenolone across the mitochondrial membrane to the smooth endoplasmic reticulum where it is converted to P_4.

Lipoproteins

Additional cholesterol is available to the luteal cell via circulating high density and low density lipoproteins (HDL's and LDL's) (Pate and Condon, 1982) or de novo synthesis from acetate (Hellig and Savard, 1965). The latter is not a major source of cholesterol unless the cell is deprived of lipoproteins and cholesterol esters (Christie et al., 1979). Lipoproteins bind to the membranes of human fibroblasts at an area of high receptor concentration, denoted "a coated pit", which is then internalized via endocytosis (Brown and Goldstein, 1976, 1983). Gwynne and Strauss (1982) found that exogenous lipoproteins are a major source of cholesterol substrate and can be used to drive steroidogenesis in luteinized rat ovaries. Similar results were found in cultured bovine luteal cells, where exogenous lipoproteins caused an increase in P_4 production (Pate and Condon, 1982).

Desensitization and Downregulation

Luteotropic agents, such as LH and hCG, in addition to initiating steroidogenesis, regulate it through "desensitization" and "down regulation" (Ryan, 1982). Desensitization usually occurs first when the hormone-receptor complex becomes altered and uncouples from the

adenylate cyclase. Down regulation is the process by which the receptor or hormone-receptor complex is removed from the cell membrane, most likely via internalization (Hildebrandt et al., 1984). LH and hCG bind to the same receptor, and Conti et al. (1977) found that in the rat, administration of physiological amounts of hCG initiated a rapid, significant desensitization and concomitant reduction in LH/hCG receptors without change in their binding affinity. Accompanying this receptor reduction was decreased adenylate cyclase activity and reduced steroidogenic response. There is a slow recovery of steroidogenic response dependent on the reinstatement of membrane receptors in ovarian tissues (Hildebrandt et al., 1984).

Calcium

The implication that the Ca^{++} ion may regulate cell function dates to 1883 with some early studies performed by Sydney Ringer. These studies suggested that Ca^{++} may be an important regulator of cell functionality. Over the last one hundred years, the great magnitude of calcium's importance in the regulation of both electrically excitable and non-excitable cells has become evident. The role of the Ca^{++} ion in excitable cells, those which contain voltage-sensitive ion channels, appears to involve its ability to stimulate exocytosis as is the case in neuronal cells, and to stimulate contractions as is the case in muscle cells. The role of Ca^{++} in non-excitable cells involves its ability to regulate various intracellular processes as a second messenger.

The movement of Ca^{++} across cell membranes is regulated by Ca^{++} channels and to this date, 4 types have been identified: 1) leak channel- the membrane is not completely imperme- able to Ca^{++} 2) stretch sensitive channel- mechanical activation allows Ca^{++} entry 3) voltage

sensitive channels- a change in membrane potential triggers the opening of these channels 4) receptor operated- ligand binding triggers opening (Schramm and Towart, 1985). Some endocrine tissues, for example rat luteal cells, are generally considered non-excitable as they do not appear to have voltage sensitive channels for Ca^{++} (Gore and Behrman, 1984). Bovine luteal cells are not believed to contain voltage-sensitive Ca^{++} channels but this fact has not been substantiated. In these cells, Ca^{++} transport across membranes occurs through 1) Ca^{++}-ATPase pump 2) Na^+/Ca^{++} exchanger and 3) voltage insensitive or receptor operated Ca^{++} channels (Carafoli, 1987; Minami and Penniston, 1987). The ability of receptor-mediated processes to regulate or mediate intracellular Ca^{++} levels is of great importance in luteal systems.

Higuchi et al. (1976) was unable to demonstrate that cells in ovine luteal tissue are electrically coupled or that LH had any effect on membrane potential. This study did show that LH was unable to stimulate P_4 in Ca^{++} depleted medium, suggesting that Ca^{++} may be important in mediating steroidogenesis although not through electrical gradients. Data from studies using adrenal tissue indicated similar results in that adrenocorticotropin (ACTH) was able to bind to its receptor but unable to stimulate adenylate cyclase in the absence of Ca^{++} (Lefkowitz et al., 1970).

Phosphoinositol Turnover

Hoken and Hoken (1953) first suggested that certain membrane phospholipids may play an important role in receptor-mediated cell signalling processes utilizing Ca^{++} as a second messenger. These phospholipids, primarily phosphatidylinositol (PI), comprise only a small portion (10%) of the total membrane phospholipid pool and are characterized by an arachidonic

acid molecule as the second functional group (Holub et al., 1970). Subsequent phosphorylations of the inositol side chain leads to the formation of the polyphosphoinositols, phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2).

Binding of the receptor agonist to the outer membrane activates the enzyme phospholipase C via a specific guanine nucleotide-dependent regulatory protein (G-protein) (Gilman, 1987). Phospholipase C acts on primarily PIP_2 yielding the hydrolyzed products, diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (IP_3) (Dawson et al., 1971). IP_3 is a water soluble product of PIP_2 hydrolysis and was suggested by Berridge (1983) as a likely candidate for the intracellular Ca^{++} mobilizing agent. IP_3 acts on the endoplasmic reticulum (ER) to induce Ca^{++} release from this non-mitochondrial pool (Spat et al., 1986). The ER is now considered the major organelle regulating cytosolic Ca^{++} levels and the major source of mobilized Ca^{++} (Exton, 1985). The mitochondria function as large Ca^{++} buffers, but do not respond to conditions resulting in transient fluctuations in intracellular Ca^{++} (Carafoli, 1987). Cytosolic Ca^{++} levels rise quickly from the resting concentration of 10^{-7} M to the activated state of 10^{-3} M. DAG then stimulates a phospholipid-dependent protein kinase, denoted protein kinase C (PK-C), by decreasing the affinity of this enzyme for Ca^{++} and results in a cascade of phosphorylations and various cell responses. PK-C is a soluble cytosolic enzyme which forms tight associations with the inner side of the lipid bilayer during activation (Takai et al., 1979b).

The presence of an active phospholipid-protein kinase C system has been demonstrated in many endocrine cells (Nishizuka et al., 1984). Stimulation by LH has been shown to cause increases in IP_3 and intracellular Ca^{++} mobilization in avian granulosa cells (Asem et al., 1987b), rat Leydig cells (Sullivan and Cooke, 1986) and bovine luteal cells (Davis et al.,

1987a). Human chorionic gonadotropin (hCG) has also been shown to elevate intracellular Ca^{++} in bovine luteal cells through an IP_3 mechanism (Davis et al., 1986). Allen et al. (1988) have shown that LH stimulates membrane PI hydrolysis, PIP and PIP_2 production and increases the levels of DAG in isolated porcine luteal membranes. These recent studies indicate that the luteotropic hormone in the bovine luteal system may be working through a Ca^{++}-PK-C modulated pathway as well as the traditional second messenger, cAMP. Ca^{++}-depleted environments have been shown to decrease stimulation of steroidogenesis in target cells by LH (Higuchi et al., 1976; and Veldhuis 1982) and ACTH (Lefkowitz et al., 1970; Farese, 1978; and Davies et al., 1985).

Other Phospholipids

PK-C has an absolute requirement for Ca^{++} and phospholipid for its activation (Nishizuka et al., 1984). DAG has been demonstrated to be the specific phospholipid required in Ca^{++} environments. However, in physiological intracellular Ca^{++} concentrations, only one phospholipid, phosphatidylserine (PS), has shown a positive effect on PK-C (Takai et al., 1979a). Other phospholipids tested, phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) were ineffective. However, the addition of PE to PS *in vitro* yielded an additional increase in PK-C activity indicating a cooperative response.

The architecture of the bilipid membrane is such that PS is found on the inner cytosolic layer and subsequent methylations of this phospholipid yield PE and PC distributed towards the extracellular side of the plasma membrane. This physical location of phospholipids appears to have physiological relevance in the activation of PK-C.

Axelrod and Hirata (1982) suggested that methylation of PE by membrane phospholipid methyltransferases plays a role in some receptor-mediated signalling events. These researchers showed that ligand-receptor complexes stimulated methylation of PE to PC in mast cells. This conversion leads to changes in the viscosity of the membrane, liberated arachidonic acid, increased Ca^{++} influx, increased phospholipase A_2 which stimulates PG production and ultimately induced histamine release. The membrane methylation of PE to PC has been coupled with an influx of Ca^{++} for transmembrane signalling (Maeda et a., 1984). Similar mechanisms may be found for other receptor-mediated processes and may operate in luteal cells.

Calmodulin

Calmodulin is a ubiquitous cytosolic protein (MW=17,000) that has been highly conserved phylogenetically. The primary function of calmodulin is to act as a calcium binding protein (Teo and Wang, 1973). The calmodulin molecule, bound with 4 Ca^{++} ions, is thought to regulate 1) many enzyme complexes 2) Ca^{++} transport 3) receptor-mediated endocytosis and 4) assembly-disassembly of microtubules (review, Cheung, 1980).

Calmodulin is present in endocrine cells and appears to have several functions. Calmodulin has been proposed to regulate the enzymes phosphodiesterase and adenylate cyclase (Chueng, 1980) in the adrenal as well as transport of cholesterol to mitochondria in adrenal tumor cells (Hall et al., 1981). The role of calmodulin in the luteal cell is not completely defined.

Cytosolic Regulation

Steroidogenesis may also be regulated at various steps beyond the plasma membrane. Protein synthesis is necessary for P_4 production. Hermier et al. (1971) showed that inhibition of ovarian protein synthesis using cycloheximide halted LH or cAMP-stimulated P_4 production within 30 minutes. This study also implicated oxygen as a regulator of steroidogenesis in that O_2 deprivation interrupted P_4 production. Oxygen is a necessary component in the P_{450} conversion of cholesterol to pregnenolone.

LUTEOLYSIS

Luteolysis, the senescence of the CL, occurs in normally cycling females if conception has not occured. The circumstances surrounding the luteolytic process are well documented for many species (Horton and Poyser, 1976; Behrman et al., 1979 and Niswender, 1981), but the mechanism of action is not at all clear.

Luteolysis, like luteotropism, is composed of functional and morphological aspects. Functional regression in most species occurs rather quickly after the onset of luteolysis, and is characterized by a dramatic drop in circulating P_4. Whether this drop results from decreased synthesis, secretion or both is not known at this time.

Morphological Luteolysis

Morphological aspects of luteolysis become evident subsequent to functional luteolysis, approximately 12 to 24 hours after the onset of regression in the rat (Fuchs et al., 1974), the

ewe (Umo, 1975) and the cow (Hansel et al., 1973). Structural changes in luteal cells of the ewe include a decrease in the amount of smooth endoplasmic reticulum, an increase in the number of membrane bound granules, a rounding of mitochondria and an accumulation of lipid droplets (Umo, 1975).

Shortly after the initiation of regression, certain morphological events such as increased numbers of coated vesicles and dense bodies in the luteal cell cytoplasm were noted in both normal and $PGF_{2\alpha}$-induced luteolysis (McClellan et al., 1977). Twenty-four hours later, the appearance of increased numbers of autophagosomes or secondary lysosomes, responsible for the degradation of intracellular components, marked the full instatement of luteolysis. Also in the cow, luteolysis is characterized by rounding of cells, cytoplasmic condensation (Hansel et al., 1973) and a loss of cytoplasmic granules which are believed to be involved with P_4 storage and release (Heath et al., 1983).

In the pig, morphological luteolysis is first indicated by an increase in coated vesicles which are related to protein uptake and probably contain hydrolases. Secondly, there is an increase in cytoplasmic dense bodies (secondary lysosomes). It is believed that this increase causes fragility within the luteal cells and eventually destruction of the tissue (Cavazos et al., 1969).

Functional Luteolysis

It has been known for some time now that normal regression of the CL requires the uterus and that removal of this tissue will induce a prolonged luteal phase (Loeb, 1923; Wiltbank, 1956). The uterus is an integral component of reproductive regulation in the ewe

(Kiracofe et al., 1966, Ginther, 1974a) and cow (Hansel, 1973; Ginther and Del Campo, 1974b). These researchers found that ligation of utero-ovarian blood vessels ipsilateral to the CL preserved function and morphology, while ligation of those on the contralateral side did not interrupt the normal luteolytic procedures. It was then proposed that a uterine factor was somehow initiating luteolysis either by a direct or indirect mechanism.

Prostaglandin $F_{2\alpha}$

Prostaglandins are 20-carbon fatty acid metabolites of arachidonic acid. Liberated by phospholipase A_2, arachidonic acid metabolites are local hormones which act as local regulators. Since prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was found in the ewe to be an abundant uterine prostaglandin and 20-40% of ovarian blood is of uterine origin, it has been suggested to be the luteolytic agent (Babcock, 1966; Pharriss and Wyngarden, 1969; Ford and Chenault, 1981).

$PGF_{2\alpha}$ and Blood Flow. Pharriss et al. (1970) in their study of the luteolytic mechanism in rats and rabbits found that $PGF_{2\alpha}$ is a potent venoconstrictor, and administration via the inferior vena cava resulted in a significant reduction of blood flow in the utero-ovarian vein. Similar research indicated that $PGF_{2\alpha}$ induced a redistribution of blood to the ovary (Novy and Cook, 1973). Total blood volume was found not to change but the amount of flow to the CL decreased while the amount to the interstial and follicular tissue increased. $PGF_{2\alpha}$ constriction of the afferent blood vessels or the small arteriovenous anastomoses of the CL is thought to initiate the redistribution. Alteration in blood flow stimulated by $PGF_{2\alpha}$ has also been suggested in sheep as a luteolytic mechanism (Baird, 1974). Two hours after the start of $PGF_{2\alpha}$ infusion, P_4 fell to 50% of controls and ovarian blood flow dropped 10-15%. Although

$PGF_{2\alpha}$-induced reduction of blood flow appears luteolytic, this effect was noted only during infusion and normal blood flow was noted when luteolysis was complete. Baird (1974) suggested that the luteolytic effect of $PGF_{2\alpha}$ was due only to the early inhibition of luteal blood supply.

Morphological changes of blood vessels during regression in sheep include obstruction of capillaries with cellular debris and degeneration of luteal vessel walls (O'Shea et al., 1977). These characteristics of regression are believed to be effects of $PGF_{2\alpha}$ from the endometrium.

Countercurrent Theory. McCracken et al. (1970) demonstrated that $PGF_{2\alpha}$ was indeed a good candidate for the inducer of luteolysis in the ewe. These researchers infused $PGF_{2\alpha}$ into the ovarian artery of ewes and induced complete luteal regression within 3-6 hours. The pathway of $PGF_{2\alpha}$ from the uterus to the ovary was uncertain, for prostaglandins in the general circulation were found to be destroyed in the lungs of the dog, rat, rabbit and guinea pig (Piper et al., 1970). At this time McCracken proposed a countercurrent theory of exchange to explain the seemingly impossible transfer of $PGF_{2\alpha}$ from the uterus to the ovary. Prostaglandin produced in the endometrial lining of the uterus is proposed to travel out the utero-ovarian vein and be transferred into the convoluted ovarian artery lying just above it. The theory has met with much criticism, but although others are quick to discredit this possibility, no other suggested alternative has been proven to be the mechanism. The most striking fault with the countercurrent theory lies in the virtual inability of substances to cross from a slow moving vein to a fast flowing artery. As Coudert et al. (1974a) point out, the physiology of the vessels does not favor such a transfer. In trying to prove the countercurrent theory, researchers have injected radiolabelled substances into the uterus or uterine veins and monitored their travels as if they

were a luteolytic substance. Studies completed using labelled xenon gas (^{133}Xe) and radioiodi-nated human serum albumin (^{131}RISA) show the difficultly in diffusion of any substance from a vein to an artery. ^{131}RISA when injected into the uterine vein was undetectable in the utero-ovarian vein or ovarian artery indicating that this large molecule was incapable of crossing the vessel walls. ^{133}Xe, in small amounts, was found in the ovarian vein 20 seconds after injection (Coudert et al., 1974a). The crossing of ^{133}Xe was explained by diffusion of the gas, not by the vessels' ability to allow passage of substances. Einer-Jensen (1974) claims that labelled xenon gas (^{133}Xe) injected directly into a uterine horn of mice, hamsters or rats resulted in a higher concentration being detected in the ipsilateral ovary than in the contralateral one. It was therefore assumed that ^{133}Xe reached the ipsilateral ovary via a utero-ovarian anastomosis. In a second study by Coudert in the same year, he presented data which strengthened the opposi-tion to the countercurrent exchange hypothesis. Injection of radioactive $PGF_{2\alpha}$ into the uterine vein resulted in no transfer to the ovarian artery indicating that in sheep, $PGF_{2\alpha}$ is not luteo-lytic via a countercurrent mechanism (Coudert et al., 1974b).

Hixon and Hansel (1974) and McCracken et al. (1972) tried a similar procedure to deter-mine the path of uterine $PGF_{2\alpha}$ in cattle. $PGF_{2\alpha}$ was injected into the lumen of a uterine horn and subsequent jugular and ovarian artery blood samples were taken. These results opposed those of Coudert in sheep, and supported the countercurrent theory. Separation of the ovarian artery and the utero-ovarian vein should result in extended cycles, if luteolysis is mediated through countercurrent transfer of $PGF_{2\alpha}$; and this was found to be the case in ewes (Baird and Land, 1973; Barrett et. al., 1971) and in guinea pigs (Ginther, 1969). But conflicting data were found in similar studies in pigs (Harrison and Heap, 1972) and sheep (Restall et. al., 1973). In

this second study Restall and colleagues surgically separated the utero- ovarian vein and the ovarian artery and infused $PGF_{2\alpha}$ into the utero- ovarian vein. Peripheral P_4 levels were below 0.5 ng/ml indicating luteolysis in both control and experimental animals.

More recent studies have suggested an alternative blood route for the transfer of $PGF_{2\alpha}$ (Alwachi et al., 1981). Instead of direct transfer from the utero-ovarian vein to the ovarian artery, these workers suggest that $PGF_{2\alpha}$ passes from the oviducal vein to the ovarian vein. Transfer from this vessel to the ovarian artery is more hemodynamically plausible.

Lymphatics. Alternate studies implicating the involvement of lymphatics in luteolysis have been carried out, mainly in sheep. An early study by Morris and Sass (1966) describes the lymphatics of the sheep as a profuse network. The flow of lymph averaged 4.15 ml/h and even reached 14.9 ml/h for one ewe. These results indicate that uterine $PGF_{2\alpha}$ may be transferred via uterine and ovarian lymphatics.

The CL is one of the most vascularized organs and capillaries which service the CL are extensive and may contribute to the large lymph flow (Morris and Sass, 1966). The morphology of the capillary endothelium was discontinuous with large gaps in the basement membrane. In certain places it was even noted that extensions of the luteal cell were present in the capillary lumen (Morris and Sass, 1966). Morris also suggested that P_4 may induce the increased frequency of endothelial gaps around luteal cells. Just prior to luteolysis, when P_4 levels are the highest, the formation of gaps could increase $PGF_{2\alpha}$'s accessibility to the luteal cells. A recent study in sheep explored the role of lymphatics which drain from the uterus to the utero-ovarian pedicle and alongside the uterine artery. The relationship and permeability of utero-ovarian lymphatics and the ovarian artery are such that transfer of a uterine luteolysin is very

possible (Staples et al., 1982). A study by Kotwica (1980) found that administration of $PGF_{2\alpha}$ to the contralateral uterine vein in pigs resulted in luteolysis implicating possible involvement of lymphatic circulation.

$PGF_{2\alpha}$ Sites of Action

Luteal Membrane. The site of prostaglandin action in luteal cells is highly controversial. Receptors for $PGF_{2\alpha}$ have been identified in equine and bovine corpora lutea (Mattioli et al., 1983). $PGF_{2\alpha}$ has been proposed to work on several cellular components. The most supported theories state that $PGF_{2\alpha}$ somehow alters the luteal cell membrane, thereby impairing the luteinizing hormone-adenylate cyclase-cAMP (LH-AC-cAMP) trigger system for steroidogenesis. A study by Agudo et al. (1984) noted that in ewes, luteal adenylate cyclase activity and LH-activated adenylate cyclase were significantly reduced 2 hours post-$PGF_{2\alpha}$ treatment. This inactivated adenylate cyclase complex may be the reason for the initial drop in plasma P_4. Lahav et al.(1976) found that $PGF_{2\alpha}$ incubated *in vitro* with rat luteal cells caused cAMP stimulated by LH to return to basal levels. It was, therefore, concluded that prostaglandin may act by interfering with LH-stimulated cAMP accumulation or by increasing cAMP degradation.

It was proposed that $PGF_{2\alpha}$ may decrease LH-binding to membrane receptors (Grinwich et al., 1976a; Luborsky et al., 1984). The ability of LH to bind to its receptor has been found to decrease as the CL ages in the ewe (Evrard- Herouard et al., 1981; Rao, 1983). Loss of the luteal membrane-bound LH receptors at the time of regression has also been implied to be the result of $PGF_{2\alpha}$ exposure in the pig (Barb et al., 1984) and in the rat (Grinwich et al., 1976a).

The study showed that $PGF_{2\alpha}$ not only depressed serum P_4 in pregnant rats, but also decreased LH receptors by as much as 72 percent. However, the drop in P_4 was noted to occur 8 hours prior to a significant reduction in hCG-LH receptors, and 12 hours prior to the receptor decrease in a similar study by Spicer et al. (1981), indicating that the actual reduction of receptor numbers may not be the initial luteolytic event. Both studies imply that receptor loss, although not the initial impetus, would insure completion of luteolysis.

Gel Phase. Other $PGF_{2\alpha}$-mediated membrane effects such as a change in phospholipid composition, have been implicated in the luteolytic process (Buhr et al., 1979). During both spontaneous and $PGF_{2\alpha}$-induced regression, rat luteal membranes have been noted to undergo a phase change from a liquid-crystalline state to a more gel-like composition. This gel phase is thought possibly to impair the movement of substances through and within the plasma membrane, and membranes of various cellular organelles. Goodsaid-Zalundo et al. (1982), found that bovine luteal cells exposed to $PGF_{2\alpha}$ develop gel-like plasma membranes after 24 hours that are far more rigid than for those not exposed. These results infer that during regression the gel phase impairs the lateral movements of the LH receptor and adenylate cyclase within the plasma membrane, hence inhibiting their coupling and interrupting steroidogenesis. It has also been suggested that regressing bovine luteal membranes undergoing a phase change experience detrimental alterations in enzyme activity, active transport and ionic balance (Carlson et al., 1982).

Several researchers have shown that a gel phase occurs at the time of regression in the rat (Buhr et al., 1979 and Carlson et al., 1981). In these studies, isolated microsomal membrane fractions were observed to contain plasma membrane and endoplasmic reticulum fractions. X-

ray diffraction of these samples revealed at 24, 48 and 72 h post-$PGF_{2\alpha}$, a lipid band with a Bragg spacing of 4.15 A characteristic of the gel phase. In Carlson's study (1981), regressing luteal microsomal membranes developed gel-like composition when exposed to a transitional temperature of 20°C at 72 h post-$PGF_{2\alpha}$. This was denoted the transitional temperature, and was found to be much lower in those membrane fractions not exposed to $PGF_{2\alpha}$. Unfortunately, gel phase is not noticed until 24 h after the start of luteolysis, and the question remains- is this a cause or an effect of $PGF_{2\alpha}$-induced regression? By twenty-four hours, spontaneous or induced regression is considered complete, and the possibility must be considered that the gel phase is nothing more than a morphological result of regression.

Na^+-K^+-ATPase. Further evidence supporting the membrane as a site of action for $PGF_{2\alpha}$ was presented by Kim and Yeoun (1983). The activity of rat luteal Na^+-K^+-ATPase was used as an indication of membrane functionality. *In vitro*, $PGF_{2\alpha}$ was found to decrease Na^+-K^+-ATPase activity in luteal slices. Following *in vivo* exposure to $PGF_{2\alpha}$, the Na^+-K^+-ATPase activity of isolated luteal membranes was not changed after treatment with $PGF_{2\alpha}$. Kim and Yeoun suggested that $PGF_{2\alpha}$ may work intracellularly to induce a reduction of membrane enzymes.

Calcium. $PGF_{2\alpha}$ is thought also to possibly act within the membrane to enhance the levels of ionic calcium (Ca^{++}) which would inhibit coupling of the LH-receptor to adenylate cyclase (Behrman et al., 1979). This theory has been challenged by data in the rat which states that in calcium-free media or in the presence of a known Ca^{++} blocking agent, $PGF_{2\alpha}$ was fully effective as indicated by decreased cAMP production (Lahav et al., 1983). Also, using radioactively labelled Ca^{++}, it was shown that $PGF_{2\alpha}$ does not stimulate Ca^{++} uptake in the

membrane. A study by Gore and Behrman (1984) suggested that Na^+ influx during luteolysis may trigger a secondary Ca^{++} influx leading to inhibition of the LH-receptor complex activation of adenylate cyclase and result in the subsequent drop in P_4 production.

Recent studies in bovine luteal cells (Davis et al., 1987b) and swine ovarian cells (Veldhuis, 1987) indicate that $PGF_{2\alpha}$ stimulates phosphoinositide hydrolysis resulting in increased IP's (IP, IP_2 and IP_3). The later study also showed $PGF_{2\alpha}$ liberating arachidonic acid, DAG and translocating cytosolic PK-C to the inner plasma membrane. These data suggest $PGF_{2\alpha}$ acts via a Ca^{++}-PK-C pathway. In support of this theory, Baum and Rosberg (1987) demonstrated that phorbol 2-myristate 13-acetate (PMA), a phorbol ester, was able to mimic the actions of $PGF_{2\alpha}$ in isolated rat luteal cells. Phorbol esters are known to freely transverse the plasma membrane and directly activate PK-C.

Cholesterol Esterase. Intracellular actions of $PGF_{2\alpha}$ have also been implicated in luteal regression. Cholesterol esterase (CE), is the enzyme which converts cholesterol esters within lipid droplets to free cholesterol and free fatty acids. Mobilization of this free cholesterol is necessary for the transfer to the mitochondria and the entrance into the cytochrome P_{450} cycle. Another enzyme, 3β -hydroxysteroid dehydrogenase (3β), is responsible for the conversion of pregnenolone (P_5) to P_4 once P_5 leaves the mitochondria. Both of these enzymes are essential to functional luteal steroidogenesis. Dwyer and Church (1979), found that *in vivo* administration of $PGF_{2\alpha}$ to rats caused a decrease of CE and 3β therefore limiting the constituents needed for P_4 synthesis. Behrman et al. (1971a) showed that injections of $PGF_{2\alpha}$ in rats induced a reduction of ovarian cholesterol esters by 75% and a dramatic suppression of cholesterol ester synthetase. It was suggested that the loss of cholesterol ester synthetase

activity lead to decreased stores of cholesterol esters hence less conversion to free cholesterol and lowered levels of P_4.

20α-ol and 20β-ol. The mechanism of $PGF_{2\alpha}$ initiation of CL regression with its concomitant drop in P_4 has been suggested to possibly involve stimulating the conversion of P_4 to a weak metabolite. Most studies supporting this hypothesis have been carried out in the rat, and examine the levels of the progestin 20α-hydroxy-4-pregnen-3-one (20α-ol). The results show within 6-12 hours of $PGF_{2\alpha}$ injection a dramatic increase in 20α-ol and corresponding decreases in P_4 (Pharriss and Wyngarden, 1969). Subsequent studies have found $PGF_{2\alpha}$ to induce an increase in 20α-ol levels both *in vivo* and *in vitro* (Behrman et al., 1971b, 1971c; Hall and Robinson, 1978; DeLa Llosa-Hermier et al., 1979 and Henderson et al., 1983).

The enzyme, 20α-hydroxy-steroid dehydrogenase (20α-OH-SDH), which is responsible for the conversion of P_4 to 20α-ol, was found to increase in activity in the last trimester of pregnancy in the rat. At the same time, P_4 levels were dropping and 20α-ol levels were rising, indicating that the enzyme becomes more active near the time of regression (Lacy et al., 1976). However, the levels of 20α-ol did not increase until several days after the initial drop in P_4 and Lacy and co-workers, believe that regression cannot be totally due to the action of 20α-OH-SDH or increased production of 20α-ol.

Torjesen et al. (1978a) stated that $PGF_{2\alpha}$ did indeed decrease P_4, but increases in 20α-ol occurred only in the presence of prolactin (PRL), a rat luteotropin. The activity of 20α-OH-SDH was later found to be PRL-dependent in the rat. P_4 suppresses PRL production, hence causing the reduced activity of the 20α-enzyme. The removal of P_4 at regression and the exposure to ovarian E_2 may stimulate PRL and increase 20α-ol (Loewit and Zambelis, 1979).

Another study by Torjesen et al. (1978b) showed that as well as increasing 20α-ol, $PGF_{2\alpha}$ induced a decrease in LH-binding sites. The loss of binding sites was considered a consequence of $PGF_{2\alpha}$ rather than a mediator of luteolytic action. P_4 conversion to 20α-ol in the rat was strongly supported by Torjesen as the main luteolytic mechanism of $PGF_{2\alpha}$.

In the cow, 20β-hydroxy-4-pregnen-3-one (20β-ol) is the primary P_4 metabolite, and its presence in the bovine CL is well documented (Hayano, et al., 1954 and Gorski et al., 1958). Although far less research has been done, an involvement in regression similar to 20α-ol has been suggested for 20β-ol (Staples and Hansel, 1961). This study indicated that oxytocin injected into heifers caused a decrease in luteal P_4 as well as an increase in 20β-ol. It has since been documented that oxytocin induces an increase in $PGF_{2\alpha}$ (Sharma and Fitzpatrick, 1974 and Mitchell et al., 1975), which could be responsible for the conversion of P_4 to 20β-ol.

Other Luteolytic Interactions

Estrogen. Estrogen (E_2) has been implicated to be luteolytic or aid somehow in the luteolytic process in the cow (Wiltbank, 1966), ewe (Hawk and Bolt, 1970; Cook et al., 1974) and human (Gore et al., 1973). In the rabbit, E_2 normally is luteotropic but when given in larger doses, results in a decrease in LH receptors as well as LH- and isoproterenol-stimulated luteal adenylyl cyclase (Kirchick and Birnbaumer, 1983). Results have also indicated that E_2 could induce a loss of LH receptors in rat Leydig cells (Hsueh et al., 1978). The ability of a supplemental luteotropin to override the luteolytic effects of E_2 has been demonstrated in the hamster (Greenwald, 1986) and the macaque (Westfahl et al., 1984).

An early study by Hixon et al. (1975) indicated that E_2 may be indirectly involved in $PGF_{2\alpha}$-induced regression. Ovaries of ewes were irradiated with a sub-lethal dose of X-rays which destroyed ovarian follicular production of E_2. Later, treatment with $PGF_{2\alpha}$ (3.5 mg) induced regression in all control ewes, but luteolysis did not occur in 4 of 5 X-ray treated animals. When E_2 was given to irradiated ewes, luteolysis occurred in all, but only after 54 hours. E_2 administration with $PGF_{2\alpha}$ resulted in regression in all cases and occured much faster than in any other treatment. Intrafollicular E_2 was suggested to enable $PGF_{2\alpha}$ to be luteolytic. Two years later, data was generated suggesting that although E_2 may in some way be luteolytic, and the effectiveness of $PGF_{2\alpha} + E_2$ was independent of the uterus, ovarian follicles are not an absolute requirement for $PGF_{2\alpha}$-induced luteolysis because an increased dose of $PGF_{2\alpha}$ (7.0 mg) *in vivo* alone to irradiated animals did cause regression (Gengenbach et al., 1977). A similar study in the cow indicated that follicular destruction via irradiation would extend cycle length (Villa-Godoy et al., 1985). These authors implied that the absence of E_2, the main steroidogenic product of follicles, was responsible for maintaining corpora lutea. The cow has potential ovulatory follicles present on the ovary on most days of the cycle, which may be responsible for supplying E_2 to initiate luteolysis by stimulating $PGF_{2\alpha}$ (Ireland et al., 1984).

Recent data further links E_2 and the stimulation of uterine $PGF_{2\alpha}$ in the initiation of regression in the bovine (Thatcher et al., 1986). More specifically, Franchi et al. (1985) suggested that E_2 activates a uterine enzyme, 9-ketoreductase, responsible for increased conversion of PGE_2 to $PGF_{2\alpha}$. PGE's are known luteotropins and can inhibit the luteolytic action of E_2 in the ewe (Hoyer et al., 1985; Weems et al., 1985), therefore, the increased conversion of PGE's

to $PGF_{2\alpha}$ would favor luteal regression.

Sotrel et al. (1981) demonstrated that E_2 plays a role in primate luteolysis. *In vivo* injections of E_2 directly into the CL were found to cause increased synthesis of ovarian $PGF_{2\alpha}$. When $PGF_{2\alpha}$ and E_2 were administered together and then the CL removed, a significant decrease in LH/hCG receptors was noted. Sotrel et al. (1981) suggested that estrogen is luteolytic in the primate via depression of LH receptors and $PGF_{2\alpha}$ works by altering the cyclic guanosine monophosphate (cGMP) system.

Treatment with E_2 *in vivo* was found to inhibit *in vitro* LH- stimulated steroidogenesis in bovine luteal cells (Hansel et al., 1973) and in luteal cells from pregnant sows (Grazul et al., 1986). Contradictory to these results, a later paper by Hixon and Hansel (1979) showed that $PGF_{2\alpha}$ stimulated P_4 accumulation in bovine luteal cell cultures, while E_2 was incapable of halting LH-stimulated P_4 production. These researchers suggested that the luteolytic effect of E_2 in the cow was not due to a direct action on the CL. Other studies have indicated a direct action of E_2 on luteal cells. Kimball and Hansel (1974) demonstrated a high affinity E_2 binding protein in the cytosol of bovine corpora lutea. A later study showed decreased ability of LH to stimulate P_4 production *in vitro* when bovine luteal cells were incubated with E_2 (Williams and Marsh, 1978). Studies using rat leydig cells indicate that E_2 blocks the androgen pathway, and causes a reduction in microsomal P_{450} (Dufau et al., 1982). Possibly, indirect and direct actions of E_2 are important in decreasing steroid production in regressing bovine luteal cells.

Oxytocin. Oxytocin is a major neuropeptide synthesized in the hypothalamus, stored in the posterior pituitary and secreted into the peripheral blood system. The major target organ of

oxytocin appears to be the uterus, and it is thought to induce labor at or near the time of parturition (Roberts et al., 1976a and Fuchs, 1983).

Evidence has been presented to suggest that oxytocin is involved in luteal regression in cycling dairy heifers (Armstrong and Hansel, 1959). Administration of oxytocin 1-7 days post-estrus significantly shortened the estrous cycle length and subsequent CL's were below normal size and cell number. Oxytocin was found to enhance the release of $PGF_{2\alpha}$ from the endometrium of sheep (Roberts et al., 1975, 1976b), the rabbit (Small et al., 1978) and the cow (Sharma and Fitzpatrick, 1974 and Mitchell et al., 1975). Small et al. (1978) presented evidence which suggests that E_2 stimulates release of oxytocin, which in turn increases the release of uterine $PGF_{2\alpha}$. Further research on this theory suggested that E_2 caused the appearance of oxytocin receptors in the uterus. Once the presence of these receptors was evident, the endogenous levels of oxytocin were capable of inducing an increased $PGF_{2\alpha}$ secretion from the uterus (McCracken, 1980). Subsequent research from the same laboratory has shown that five 1 hour pulses of $PGF_{2\alpha}$ triggered by OT from the pituitary resulted in an increased production of luteal OT. This burst of luteal OT stimulates the luteolytic dose of uterine $PGF_{2\alpha}$ (Lamsa and McCracken, 1986).

Recently, the presence of oxytocin in the CL and the involvement in luteal regression of cycling animals has been proposed for sheep (Flint and Sheldrick, 1983), pigs (Wathes and Swann, 1982), humans (Dawood and Khan-Dawood, 1986) and cows (Fields et al., 1983; Schallenberger et al., 1984). Harrison et al. (1987) demonstrated OT production by only large ovine cells or mixed populations of small and large cells. OT was produced only during the early and midcycle stages and was undetectable later in the cycle. It has been suggested that

the sheep CL is capable of producing 50% of the total amount of systemic oxytocin present in the body and this positively feeds back to increase uterine production of $PGF_{2\alpha}$ and further ovarian oxytocin, resulting in regression (Flint and Sheldrick, 1983).

Fields et al. (1983) discovered a "contractin factor" in human placental tissue and bovine corpora lutea, which has been identified as oxytocin or oxytocin-like. The virtual absence of OT mRNA in luteal tissue from pregnant cows further suggests the role of OT in initiating luteolysis (Ivell et al., 1985). If the bovine CL is producing sufficient oxytocin, then OT-dependent release of $PGF_{2\alpha}$ may be an important initial mechanism of regression.

GnRH. Gonadotropin-releasing hormone (GnRH) has long been known for its indirect action on the gonads via LH and FSH released from the anterior pituitary, but more recent evidence supports possible direct actions of GnRH on rat ovary (Pieper et al., 1981), granulosa cells (Jones et al., 1980) and luteal cells (Clayton et al., 1979; Harwood et al., 1980). Researchers have shown specific GnRH receptor sites in the gonads which closely resemble those in the pituitary of the rat. No such sites have been identified in monkey (Asch et al., 1981) and human luteal tissue (Clayton and Huhtaniemi, 1982), monkey and human testes (Clayton and Huhtaniemi, 1982) and ovine, bovine and porcine ovaries (Brown and Reeves, 1983).

In the rat, the role of GnRH in ovarian function appears to be primarily at the stage of follicular development. Effects have also been demonstrated in rat luteal cells showing GnRH and GnRH analogs (GnRHa) to decrease LH-stimulated cAMP and P_4 production (Behrman et al., 1980) and to initiate luteolysis (Bex and Corbin, 1979).

A study by Milvae et al. (1984) indicated that native GnRH influenced basal and LH-stimulated P_4 production in isolated bovine luteal cells only when added in extremely high doses. *In vivo* experiments from the same study were unable to show an effect of native GnRH on plasma P_4 or estrous cycle length, while a potent analog (GnRH-A-1) proved luteotropic and extended the cycle. Similar results were seen in cultured rat Leydig cells which responded to GnRHa with an increase in steroidogenesis (Moger, 1984). Studies using human corpus luteum membranes showed no effect of LHRHa on adenylyl cyclase activity (Rojas and Asch, 1985). Knecht et al. (1983) have reviewed many studies showing GnRHa to be Ca^{++} dependent, induce PI turnover and possibly work through PK-C.

The absence of GnRH receptors and effect of only high doses of native GnRH have lead researchers to conclude that this hormone may not act via a direct mechanism on the luteal cell of the aforementioned species. Rather, any antigonadotropic actions of GnRH may be mediated indirectly through elevated pituitary release of gonadotropins suppressing or downregulating luteal LH receptors.

Although the presence of GnRH in bovine and ovine corpora lutea remains undetected, a GnRH-like protein has recently been isolated from luteal tissue in these species (Ireland et al., 1987; Aten et al., 1987). This protein was capable of decreasing LH-stimulated cAMP accumulation in rat luteal cells but was not tested in a bovine or ovine luteal system. A similar protein has been isolated from human ovaries (Aten et al., in press) and suggests that the direct antigonadotropic effects are not mediated through GnRH in these species, but, through a GnRH-like factor. In the bovine, levels of GnRH-like protein increase during regression implicating involvement in this process (Ireland et al., 1987).

Nature of $PGF_{2\alpha}$ Action

The actions of $PGF_{2\alpha}$ *in vivo* and *in vitro* at this time appear conflicting. Except for a refractory period of 3 to 4 days after ovulation, *in vivo* administration of $PGF_{2\alpha}$ induces luteal regression in most species. In short term bovine luteal culture, $PGF_{2\alpha}$ has been found to be luteotropic (Speroff and Ramwell, 1970; Hixon and Hansel, 1979; Weston and Hansel, 1980), luteolytic (O'Grady, 1972) or neither (Evrard et al., 1978; Wright et al., 1980; Litch and Condon, 1988). Recently, long term culture indicates that the action of $PGF_{2\alpha}$ changes from luteotropic on the first day of culture to luteolytic from that day onward (Pate and Condon, 1984). The controversy concerning this dual nature of $PGF_{2\alpha}$ has suggested that the action of $PGF_{2\alpha}$ is dependent on the hormonal environment of the luteal cells. An early CL is refractory to $PGF_{2\alpha}$, but it is greatly increasing the number of LH-binding sites and is far more responsive to LH (Spicer et al., 1981).

It was, therefore, suggested that the maintenance of the CL and the induction of regression depends on the relative levels of luteotropin and luteolysin. It has been shown that multiple injections of hCG are capable of inhibiting the luteolytic effect of $PGF_{2\alpha}$ and postponing luteal regression in the cycling ewe (Bolt, 1979). Contradictory to these results, injections of hCG in the cow were unable to block the luteolytic actions of $PGF_{2\alpha}$ *in vivo* (Litch and Condon, 1988). Also, infusions of LH followed by $PGF_{2\alpha}$ were unable to inhibit luteolysis in the ewe (Sasser et al., 1977) and in the cow (González-Menció et al., 1977).

Exogenous hCG has been shown to inhibit luteolysis in the pig (Guthrie and Bolt, 1983) and exogenous prolactin (PRL) was capable of overriding the luteolytic effect of $PGF_{2\alpha}$ in the

rat (Grinwich et al., 1976). Torjesen and Aakvaag (1984) produced similar results in the rat with hCG and suggested that pre-treatment of hCG causes "stockpiling" of cAMP which may be the mechanism to inhibit luteolysis. This research indicates that $PGF_{2\alpha}$ induces a loss of LH-adenylate cyclase and decreases cAMP *in vivo* but the LH receptors remain active and *in vitro*, the cells could still respond to hCG and dbcAMP, although P_4 levels were below that of untreated animals.

The controversy as to the mechanism of action of $PGF_{2\alpha}$ remains unanswered. Therefore, the purpose of this study was to examine the effects of $PGF_{2\alpha}$ in a defined bovine luteal cell culture system in hopes of elucidating the mechanism of action of this endocrine regulator.

INTRODUCTION

Luteolysis, the death of the corpus luteum (CL), is a complex physiological event necessary for the maintenance of cyclicity in the reproductively mature female. Luteolysins are species-specific, endogenous compounds which cause luteal regression if fertilization does not occur during the estrous cycle. Most non-primate mammals, including the cow, respond to an arachidonic acid metabolite, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as the agent of regression. Although $PGF_{2\alpha}$ is quite active *in vivo*, inducing regression 1 h post-injection, its *in vitro* actions are highly variable. Bovine luteal cells in a 2 h incubation respond to $PGF_{2\alpha}$ by maintaining or increasing basal and inhibiting LH-stimulated P_4 production (Speroff and Ramwell, 1970; Hixon and Hansel, 1979; Litch and Condon, 1988). Luteal cells exposed to $PGF_{2\alpha}$ for longer periods of time (2-10 d) demonstrate slight decreases in basal P_4 and complete inhibition of LH-stimulated P_4 production, but never exhibit full functional or morphological regression (Pate and Condon, 1984). The mode of action of $PGF_{2\alpha}$ in the induction of bovine luteolysis is not fully understood.

3β-hydroxysteroid dehydrogenase (3β-HSD) is an enzyme present in the endoplasmic reticulum of steroidogenic cell types and is responsible for the conversion of pregnenolone (P_5) to P_4. The activity of this enzyme appears to be under hormonal regulation. Several researchers have demonstrated the ability of follicle-stimulating hormone (FSH) to stimulate increased activity of 3β-HSD in cultured rat granulosa cells (Zeleznik et al., 1974). *In vivo* studies have demonstrated significant decreases in rat ovarian 3β-HSD in response to $PGF_{2\alpha}$ administration

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(Dwyer and Church, 1979). These researchers have suggested the mode of action of $PGF_{2\alpha}$ is to block P_4 synthesis at an intracellular site, particularly at the endoplasmic reticulum.

The role of calcium (Ca^{++}) in the regulation of physiological events has long been recognized. The absence of Ca^{++} is detrimental to LH-stimulated steroidogenesis in porcine granulosa cells (Veldhuis et al., 1984), and ovine (Higuchi et al., 1976) and bovine luteal cells (Davis et al., 1987a). Luteal cells incubated for 20 minutes with the Ca^{++} chelator, EGTA, are unable to respond to LH. The possible involvement of Ca^{++} in the process of luteolysis has only recently been proposed (Lahav et al., 1983; Gore and Behrman, 1984). In the rat, $PGF_{2\alpha}$ was suggested to alter membrane flux of Ca^{++} blocking the lateral translocation of the LH-receptor complex to adenylate cyclase (Gore and Behrman, 1984).

Although the uterus is the primary site of $PGF_{2\alpha}$ production (Pharris and Wyngarden, 1969) and is mandatory for the initiation of regression (Wiltbank, 1956 and Hansel, 1973), researchers have shown endogenous production of $PGF_{2\alpha}$ by the porcine CL (Patek and Watson, 1976) and cultured bovine luteal cells (Pate and Condon, 1984). Intracellular Ca^{++} has been suggested to regulate production of $PGF_{2\alpha}$ in other cell types, such as rat (Poyser, 1985) and ovine uterine cells (Silvia and Homanics, 1987). Possibly, production of endogenous $PGF_{2\alpha}$ in bovine luteal cells is also influenced by the Ca^{++} environment. The function of this luteal $PGF_{2\alpha}$ is not completely known, but it may play a role as an autocrine regulator of P_4 production within the luteal cell.

Other studies have shown Ca^{++} is required for $PGF_{2\alpha}$ binding to plasma membranes (Deliconstantinos and Fotiou, 1986). In bovine luteal cells, $PGF_{2\alpha}$ mobilizes intracellular Ca^{++} and stimulates production of inositol phosphates via phospholipid hydrolysis (Davis et

al., 1987b). The definitive role of Ca^{++} in $PGF_{2\alpha}$-induced luteolysis remains unknown.

Calmodulin is a ubiquitous intracellular Ca^{++} binding protein which has several proposed functions including enzyme regulation of adenylate cyclase, phosphodiesterase, phospholipase A_2 and Ca^{++}-dependent protein kinase (Cheung, 1980). Calmodulin does influence steroidogenesis in endocrine cells (Hall et al., 1981), but the function of calmodulin in the regulation of luteal steroidogenesis is not understood.

Native estrogen (E_2) is luteolytic in the cow during certain phases of the estrous cycle (Thatcher et al., 1986). These researchers have proposed a cooperative effect of E_2 and $PGF_{2\alpha}$ in the initiation of luteal regression. Recently, phenol red, a pH indicator present in most commercially prepared cell culture media, has been identified as a weak estrogen. Hubert et al. (1986) have demonstrated phenol red competitively binding to the E_2 receptor in uterine cells. Studies using cultured rat anterior pituitary cells (Hofland et al., 1987) and MCF-7 cells, a human breast cancer cell line (Sheen and Katzenellenbogen, 1987) have shown decreased effects of E_2 on these target cells and have attributed this decrease to the phenol red present in the culture medium.

Bovine luteal cells cultured in Ham's F12 medium are exposed to approximately 1 ug/ml of phenol red and to 10 ug/ml if cultured in minimal essential medium (MEM). These media are used routinely for *in vitro* physiological studies of luteal function, and the phenol red present may be acting estrogenically to influence steroidogenesis in a pharmacological manner.

The purpose of this study was to examine the *in vitro* actions of $PGF_{2\alpha}$ in a bovine luteal culture system. Research was organized to investigate 1) the influence of LH and $PGF_{2\alpha}$ on 3β-HSD in bovine luteal cells 2) the role of Ca^{++} in the regulation of luteal steroidogenesis and

regression and 3) the influence of estrogen and phenol red on basal, LH- and $PGF_{2\alpha}$-induced

steroidogenesis in bovine luteal cells.

MATERIALS AND METHODS

General Methods of Tissue Collection and Dissociation

In all experiments, midcycle corpora lutea (days 9-12, estrus=day 0) were collected per vaginum from regularly cycling, non-lactating dairy cows of mixed breeds. All tissue was handled aseptically from the time of collection and kept on ice during transportation to the laboratory. Tissue was weighed, minced and enzymatically dissociated with collagenase Type I (Worthington Biomedical Corp., Freehold, NJ)) at 2000 units/g tissue according to the method of Simmons et al., 1976, as modified by Pate and Condon, 1982 (Appendix A). Cell viability was determined using a hemacytometer and the trypan blue exclusion method (Tennant, 1964). All culture media contained 24 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES buffer, Calbiochem-Behring Corp., LaJolla, CA), insulin (5 µg/ml), transferrin (5 µg/ml) and selenium (5 ng/ml) (ITS premix, Collaborative Research Inc., Bedford, MA) and the antibiotic, Gentamicin (20 µg/ml) (Gibco Laboratories, Grand Island, NY). Cultures were maintained for 8 to 10 days in a closed culture system at 37°C. Culture medium was collected and replaced every 48 h. Collected culture medium was frozen at -20°C until assayed by radioimmunoassay (RIA). Unextracted medium samples were assayed for P_4 using the RIA procedure of Pate and Condon, 1982 (Appendix B). The anti-progesterone-11-BSA serum (GDN-337) was supplied by Dr. Gordon Niswender, Colorado State University. This antiserum was not found to cross react significantly with any other steroid in the samples (Gibori et al., 1977). The labelled progesterone, $[1,2-^3H]-P_4$, was purchased from New

England Nuclear, Boston, MA. The intra-assay coefficient of variation was (8.5%) and the inter-assay coefficient of variation was (17.1%). The sensitivity of the assay was 20 pg/ml. The statistics used on the data from these studies were one-way analysis of variance (ANOVA) to identify the existence of variability, and Newman-Kuels to separate the means and determine significant treatment variations. See Appendix C for experimental design.

Experiment I

Dissociated bovine luteal cells were seeded in 6-well culture plates (Nunc,) at a concentration of 2.5 x 10^5 cells/well. The cells were cultured serum-free in Ham's F12 culture medium (Hazelton Research Products, Lenexa, KS) for 8 days with medium replacement every 48 h. Cells were treated with bovine luteinizing hormone (bLH) (NIAMMD-bLH-4, Bethesda, MD.) (10 ng/ml), prostaglandin $F_{2\alpha}$-Tris Salt ($PGF_{2\alpha}$) (Sigma P3023) (Sigma Chemical Co., St. Louis, MO) (10 ng/ml), and LH + $PGF_{2\alpha}$. Collected medium was frozen at -20°C and assayed for P_4 using using RIA. Plates were stained on days 2, 4 and 8 for 3β-hydroxy steroid dehydrogenase (3β-HSD) using the protocol of Payne et al., 1980. Briefly, medium was collected from each well and plates were washed in 0.1% phosphate buffer (PB) and fixed in 1% paraformaldehyde at 4°C for 20 min. Fixed cells were rinsed 3 times with 0.1% PB, dried, then incubated with 0.25 mM nitroblue tetrazolium (Sigma N6876) (Sigma Chemical Co., St. Louis, MO), 0.1 % bovine serum albumin (BSA), 1.5 mM-nicotinamide adenine dinucleotide (NAD) (Sigma N1511) (Sigma Chemical Co., St. Louis, MO) and 0.2 mM etiocholan-3-ol-17-one (Sigma E5251) (Sigma Chemical Co., St. Louis, MO) at 37°C for 90 min. Corresponding wells of the same treatments were also subjected to the fixation and staining processes in the

absence of etiocholan-3-ol-17-one to serve as technique controls. 3β-positive cells (3β+) and total cell populations were enumerated using an ocular grid on an inverted microscope. Cells were considered 3β+ if they stained dark blue.

Experiment II

Bovine luteal cells were seeded in 25 cm^2 culture flasks (Corning Co., Corning, NY) at a concentration of 1 x 10^6 cells/flask. Cells were cultured serum-free in Ham's F12 culture medium for 8 days with medium replacement every 48 h. All cells were seeded in Ca^{++}- containing medium to facilitate attachment to the flask substrate. Twenty-four hours post-seeding (Day 0), cells were treated with: LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), LH + $PGF_{2\alpha}$, cal-cium ionophore A23187 (10 nM) (Sigma C7522) (Sigma Chemical Co., St. Louis, MO), A23187 + LH, A23187 + $PGF_{2\alpha}$, A23187 + LH + $PGF_{2\alpha}$, ethylene glycol-bis(-aminoethyl ether) N,N,N',N'-tetra-acetic acid (EGTA) (0.3 mM) (Sigma E4378) (Sigma Chemical Co., St. Louis, MO), EGTA + LH, EGTA + $PGF_{2\alpha}$, and EGTA + LH + $PGF_{2\alpha}$. Cultures were also treated with the following intracellular Ca^{++} antagonists, 3,4,5-trimethoxy-benzoic acid 8-(diethylamino) octyl ester (TMB-8) (2 μM) (Sigma T0517) (Sigma Chemical Co., St. Louis, MO), carbonyl cyanide m-chloro phenyl-hydrazone (CCCP) (50 μM) (Sigma C2759) (Sigma Chemical Co., St. Louis, MO); calmodulin antagonists, trifluoperazine (TFP) (1 μM) (Sigma T8516) (Sigma Chemical Co., St. Louis, MO) and N-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide (W-7) (5 μM) (Sigma A3281) (Sigma Chemical Co., St. Louis, MO) and a voltage-sensitive Ca^{++} channel blocker, nifedipine (NF) (Sigma N7634) (Sigma Chemical Co., St. Louis, MO). Cells were enumerated on days 2 and 6 using an ocular grid on an inverted

microscope (Pate and Condon, 1984). Medium was collected on days 2, 4, 6 and 8 of culture and frozen at -20°C until assayed for P_4 by RIA. Medium samples were also assayed for $PGF_{2\alpha}$ using RIA (Pate, 1987). The intra-assay coefficient of variation was 9.6% and the inter-assay coefficient of variation was 15.9%. The sensitivity of the assay was 12.5 pg/ml.

Experiment III

Corpora lutea were dissociated in Ham's F12 culture medium devoid of phenol red (Gibco Laboratories, Grand Island, NY) or minimal essential medium (MEM) without phenol red (Gibco Laboratories, Grand Island, NY). Dissociated cells were seeded in 25 cm^2 culture flasks in either phenol-containing & phenol-depleted Ham's F12 or phenol-containing & phenol-depleted MEM at a concentration of 1×10^6 cells/flask. Cells were cultured in a closed culture environment at 37°C for 10 days. Cultures were treated with LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), LH + $PGF_{2\alpha}$, 17β-estradiol glucuronide (E2) (1 ng/ml) (Sigma E2127) (Sigma Chemical Co., St. Louis, MO), E2 + LH, and E2 + $PGF_{2\alpha}$. Cells were enumerated on days 2 and 8. Medium was collected on days 2, 4, 6, 8, and 10. Collected culture medium was frozen at -20°C and assayed for P_4 using RIA.

RESULTS

Experiment I

On day 2 of culture, P_4 production in response to LH-treatment (10 ng/ml), was 17.6% below control values while $PGF_{2\alpha}$ (10 ng/ml) stimulated P_4 21.4% above controls (Fig. 1). By day 4 of culture LH began to stimulate P_4 production (14.4% above controls), while $PGF_{2\alpha}$ became inhibitory (25.7% below controls). Subsequent days in culture (day 6 and day 8) showed a continued stimulation of P_4 by LH of approximately 100% above controls. $PGF_{2\alpha}$ treatment on days 6 and 8 was neither stimulatory nor inhibitory, as P_4 values remained not significantly different from controls ($P>0.05$).

During the 8 day culture period, total numbers of cells were monitored (Fig. 2). On days 2 and 4 of culture, approximately 2×10^5 cells/treatment group were maintained. By day 8 of culture all treatment groups (control, LH and $PGF_{2\alpha}$) showed a loss in total cell numbers of approximately 20% compared to days 2 and 4. At no point during the culture period were cell numbers affected by LH or $PGF_{2\alpha}$.

Throughout the 8 day culture period of this experiment, luteal cells in 6-well plates were stained for the presence of the steroidogenic enzyme, 3β-HSD (day 2, Fig. 3; day 4, Fig. 4; day 8, Fig. 5). Luteal cells which stained heavily were considered 3β-HSD positive as compared to technique controls (Fig. 6). The % 3β-HSD positive cells of the total cell population was calculated for days 2, 4 and 8 of culture (Fig. 7). On day 2 of culture all treatment groups (con-

trol, LH and $PGF_{2\alpha}$) contained approximately 80% 3β-HSD positive cells, representing the steroidogenically active portion of the cell population. As the culture progressed, all treatment groups experienced decreases in the numbers of 3β-HSD positive cells. On day 4 control cultures showed a 22% decrease and $PGF_{2\alpha}$-treated cultures showed a 31% drop in 3β-HSD positive cells. Although the LH-treated cells lost 10% of the 3β-HSD positive cells present on day 2, this drop was not significant ($P>0.05$). All treatment regimes demonstrated further losses in the 3β-HSD population by day 8, control cultures losing 61.6% of the steroidogenically active cells present on day 2 and $PGF_{2\alpha}$ losing 52.1% ($P<0.05$). LH-treated flasks showed a decrease of approximately 35% of the original, day 2 3β-HSD cells.

Experiment II

The purpose of this experiment was to investigate the influence of Ca^{++} environment on steroidogenesis of cultured bovine luteal cells. Luteal cells cultured for 2 days showed no significant changes ($P>0.05$) in P_4 production in response to LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml) or LH + $PGF_{2\alpha}$ in control, Ca^{++}-enriched (A23187, 10nM) or a Ca^{++}-depleted environments (EGTA, 0.3 mM) (Fig. 8). On day 4 of culture, LH significantly stimulated P_4 in control and A23187-treated cultures, but was unable to increase P_4 in the presence of EGTA (Fig. 9). $PGF_{2\alpha}$ lowered basal P_4 slightly on this day of culture in control, A23187 and EGTA-treated flasks, and completely inhibited LH-stimulated P_4 in all treatment groups. Subsequent days in culture (day 6; Fig. 10 and day 8; Fig. 11) showed significant ($P<0.01$) stimulation of P_4 by LH in control cultures with an additional increase in the presence of Ca^{++} ionophore (A23187). EGTA completely inhibited the ability of LH to stimulate P_4. $PGF_{2\alpha}$ decreased P_4 below

basal production in control, A23187 and EGTA treatments, although in each case this decrease was not significant (P>0.05). The ability of $PGF_{2\alpha}$ to inhibit LH-stimulated P_4 production was maintained throughout the culture period in both Ca^{++}-enriched (A23187) and Ca^{++}-depleted (EGTA) environments (P<0.01).

Levels of endogenously produced $PGF_{2\alpha}$ were measured for 8 day cultured bovine luteal cells subjected to Ca^{++}-enrichment (A23187, 10nM) or Ca^{++}-depletion (EGTA, 0.3 mM) in the presence or absence of LH (10 ng/ml). Levels of $PGF_{2\alpha}$ were detectable in the medium only through day 4 of culture. Day 2 of culture showed no significant changes (P>0.05) in production of $PGF_{2\alpha}$ in any Ca^{++} environment or in the presence or absence of LH (Fig. 12). The same was true for day 4 of culture (Fig. 13). Production of $PGF_{2\alpha}$ on this day was lower in all treatments, approximately one-third that of day 2 levels, and no significant treatment effects were noted (P>0.05).

Dose response data were generated for two intracellular Ca^{++}-antagonists TMB-8 (Table 1) and CCCP (Table 2), two calmodulin-antagonists, TFP (Table 3) and W-7 (Table 4) and one voltage-sensitive Ca^{++} channel blocker, NF (Table 5). NF treatment at 1 μM, 100 nM or 10 nM had no effect on basal P_4 production. The addition of NF (100 nM) to LH-treated cells produced P_4 levels that were not significantly different from LH treatment alone (P>0.05) (data not shown).

Selected doses of TMB-8 (2 μM), CCCP (50 μM), TFP (1 μM) and W-7 (5 μM) were added throughout 8 day cultures of bovine luteal cells in the presence or absence of LH and $PGF_{2\alpha}$. On day 2 of culture no significant treatment effects were noted in any culture condition (P>0.05)(Fig. 14). By day 4 of culture, LH significantly stimulated P_4 production

(P<0.01) (Fig. 15). $PGF_{2\alpha}$-treated flasks had P_4 levels below control values but this difference was not significant (P>0.05). Basal P_4 production was reduced in the presence of both intracellular Ca^{++} antagonists, TMB-8 and CCCP. LH-stimulation of P_4 was completely inhibited by both Ca^{++} antagonists while $PGF_{2\alpha}$-induced P_4 was slightly reduced by CCCP and uneffected by TMB-8. Calmodulin antagonists, TFP and W-7, were unable to significantly alter basal P_4 on day 4 of culture although TFP did lower P_4 levels by 32%. LH and $PGF_{2\alpha}$-induced P_4 were not effected by these drugs (P>0.05), although LH-stimulated P_4 was slightly impaired in both cases. Similar trends were seen on day 6 (Fig. 16) and day 8 (Fig. 17) of culture.

Experiment III

The purpose of this experiment was to investigate the direct effects of E_2 and phenol red on the steroidogenesis of cultured bovine luteal cells. Cells cultured in phenol-containing and phenol-free Ham's F12 medium in the presence of LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH or E_2 + $PGF_{2\alpha}$ showed no significant differences in P_4 production on day 2 of culture (Fig. 18). Although not significant (P>0.05), E_2 tended to stimulate P_4 in the presence of LH in phenol-containing medium, while inhibiting P_4 in phenol-free medium during the first 2 days of culture. On day 4, LH increased P_4 in both media, but in phenol-free cultures LH treatment yielded significant increases (P<0.01) in P_4 production with levels 47.4% above those in phenol-containing Ham's F12 (Fig. 19). E_2, $PGF_{2\alpha}$ and E_2 + $PGF_{2\alpha}$ treatment had no effect on basal P_4 in either medium (P>0.05). In Ham's F12 E_2 was unable to influence LH-induced P_4 but could significantly inhibit LH-stimulated P_4 in phenol-free cul-

tures ($P<0.01$). Day 6 cultures in Ham's F12 and phenol-free Ham's F12 responded to LH with significant increases in P_4 ($P<0.05$ and $P<0.01$, respectively), and cells in phenol-free medium again responded with increases of 20% above cultures with phenol red (Fig. 20). E_2, $PGF_{2\alpha}$ and $E_2 + PGF_{2\alpha}$ showed no differences from control values on day 6 in either medium ($P>0.05$). E_2 suppressed LH-stimulation only in phenol-containing medium. The ability of E_2 to block LH-stimulation in phenol-free medium becomes less effective as the length of culture increases (day 6, Fig. 20, day 8, Fig. 21 and day 10, Fig. 22). By day 10 of culture E_2 has no effect on LH-stimulation in phenol-free medium, as P_4 production in this treatment is no different from LH alone ($P>0.05$). E_2 in Ham's F12 slightly depressed LH-stimulated P_4 production on both day 8 and 10 ($P<0.10$). E_2, $PGF_{2\alpha}$ and $E_2 + PGF_{2\alpha}$ had no effect on basal P_4 in phenol-containing or phenol-free Ham's F12 on either day 8 or day 10 ($P>0.05$). LH-stimulation of P_4 was significantly higher than controls in both media and on both day 8 and day 10 of culture. Levels of P_4 in the phenol-free, LH-treated cultures were 43.3% higher than those in the phenol-containing medium on day 8 and 24.7% higher on day 10.

The same series of E_2 treatments were added to cells cultured in minimal essential medium (MEM) (Fig. 23). Day 2 of culture showed significant stimulation of P_4 by LH in phenol-free MEM ($P<0.01$). This does not correspond to the lack of day 2 responsiveness seen in Ham's F12 (Fig. 18). The initial response of cells to $E_2 + PGF_{2\alpha}$ in phenol-containing MEM was a significant ($P<0.01$) decrease in P_4 from controls. By day 4 of culture, LH was stimulatory in both phenol-containing and phenol-free MEM although significant in phenol-free MEM ($P<0.01$) (Fig. 24). E_2 had no effect on LH-stimulation in MEM on this day, but it slightly diminished the LH-response in phenol-free MEM ($P>0.05$). $PGF_{2\alpha}$, E_2 and $E_2 +$

$PGF_{2\alpha}$ were unable to significantly alter P_4 production ($P>0.05$).

On day 6 of culture, LH remained able to significantly ($P<0.01$) elevate P_4 in both media. LH-induced absolute levels of P_4 in phenol-free MEM were significantly higher than LH-stimulated levels in phenol-containing MEM ($P<0.01$). $PGF_{2\alpha}$, E_2 and E_2 + $PGF_{2\alpha}$ remained ineffective in altering P_4 production in both phenol-containing and -free media. Similar trends were evident on days 8 (Fig. 26) and 10 (Fig. 27), although the suppression of LH-stimulated P_4 due to phenol red was not statistically significant ($P>0.05$). E_2 remained capable of suppressing LH-stimulated P_4 in both media on days 8 ($P<0.01$) and 10 ($P<0.05$ in phenol-containing MEM and $P<0.01$ in phenol free MEM).

Throughout this MEM culture, LH (10 ng/ml) was able to stimulate absolute levels of P_4 approximately 60% higher than in Ham's F12 culture medium.

Figure 1.

P$_4$ production by 8 day cultured bovine luteal cells (expressed as % above control) for LH (10 ng/ml) and PGF$_{2\alpha}$ (10 ng/ml) treatments. Line C = control values. Bars with different superscripts are statistically different. mean±SEM, n=3.

FIGURE 1
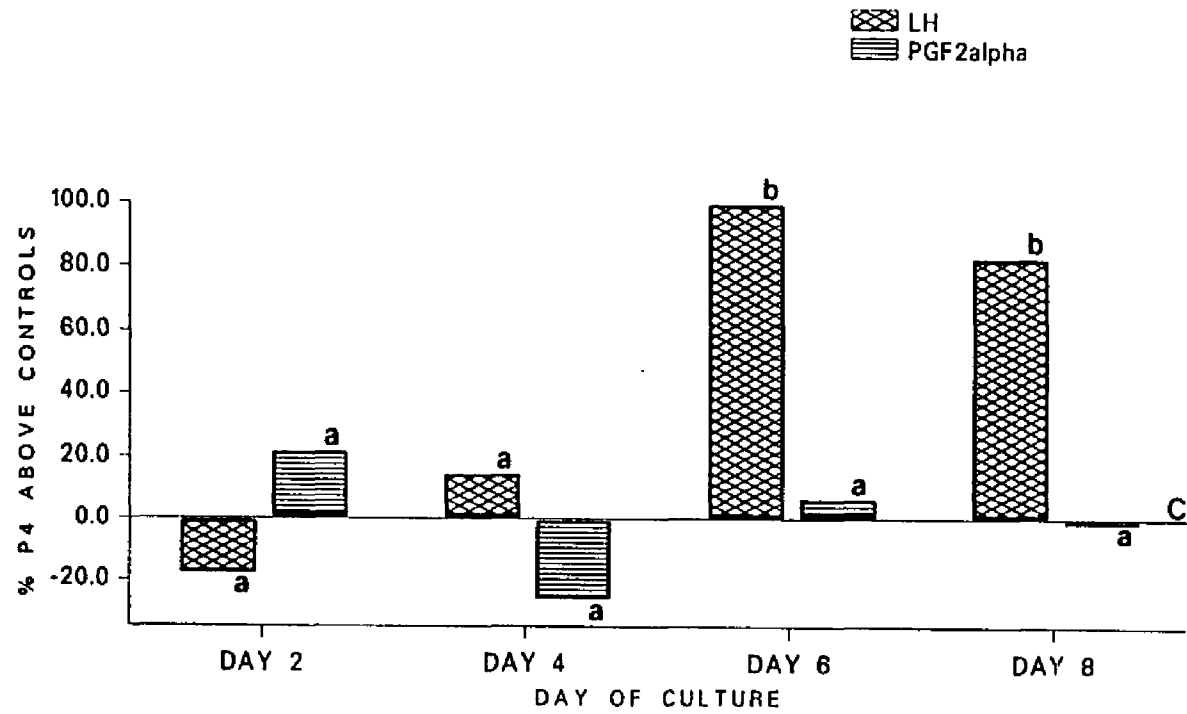
Figure 2.
Total numbers (x 1000) of luteal cells during an 8 day culture period for control, LH (10 ng/ml) and $PGF_{2\alpha}$ (10 ng/ml) treatments. Bars with different superscripts are statistically different. mean±SEM, n=3.

FIGURE 2

Legend:
- CONTROL
- LH
- PGF2alpha

Y-axis: TOTAL LUTEAL CELLS (x 1000) — 0.0, 50.0, 100.0, 150.0, 200.0

X-axis: DAY 2, DAY 4, DAY 8 — DAY OF CULTURE
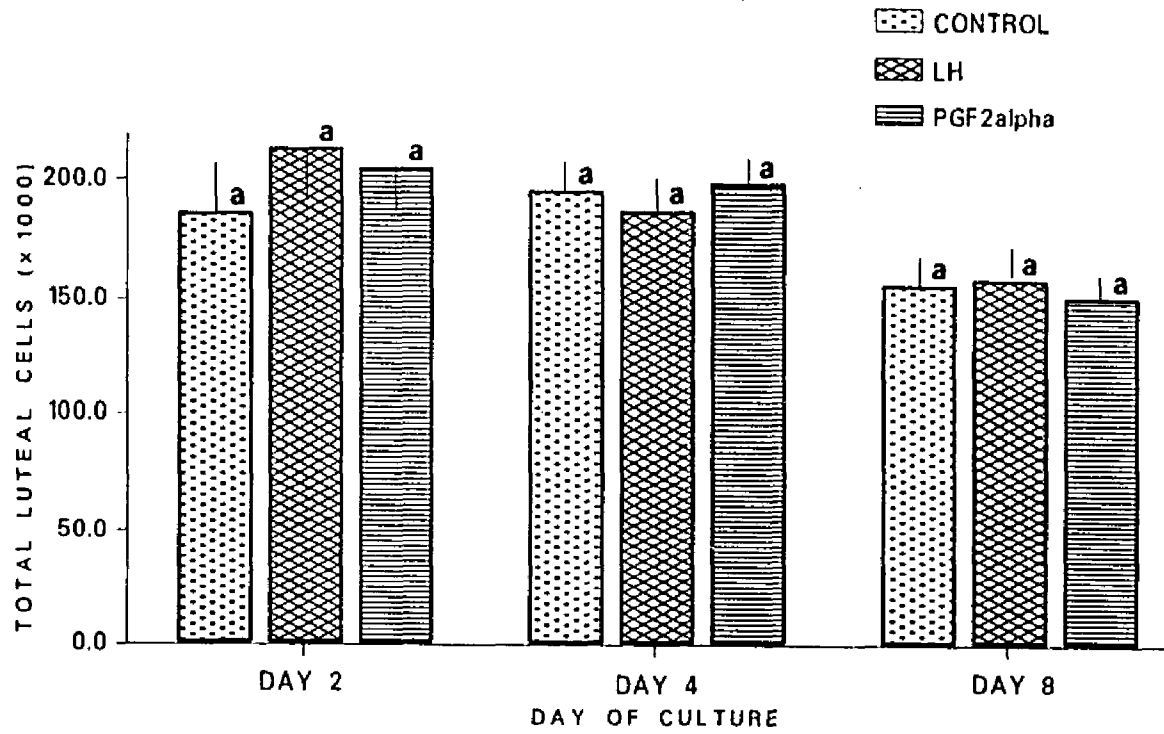
Figure 3.

3β-HSD staining in day 2 cultured bovine luteal cells for A. control, B. LH (10 ng/ml) and C. $PGF_{2\alpha}$ (10 ng/ml) treatments (×125).
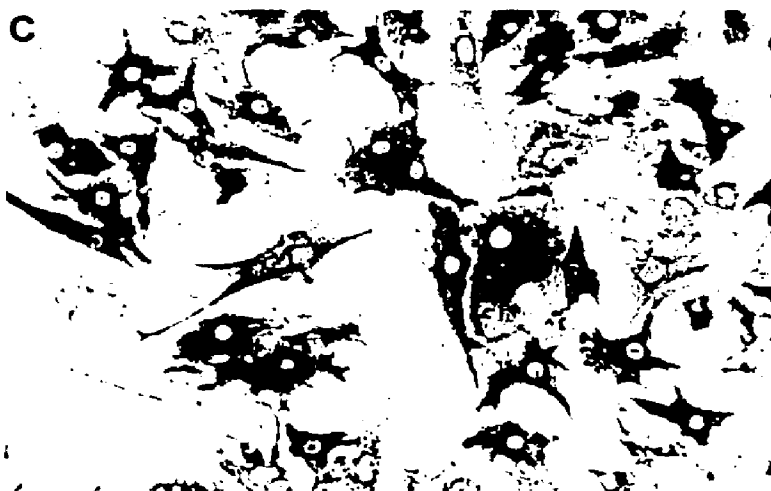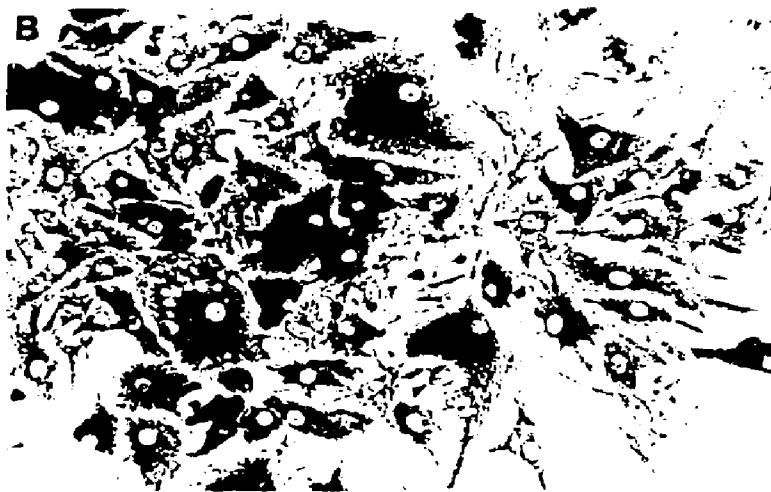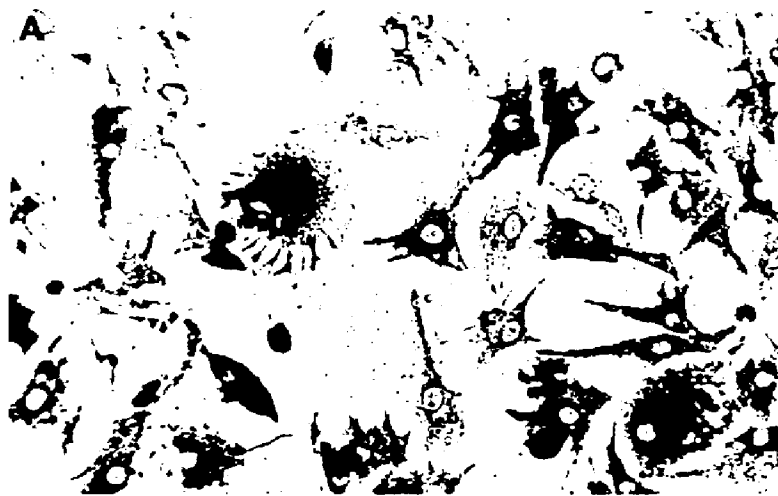
Figure 4.
3β-HSD staining in day 4 cultured bovine luteal cells for A. control, B. LH (10 ng/ml) and C. PGF$_{2\alpha}$ (10 ng/ml) treatments (×250).

Figure 5.
3β-HSD staining in day 8 cultured bovine luteal cells for A. control, B. LH (10 ng/ml) and C. PGF$_{2\alpha}$ (10 ng/ml) treatments (×145).
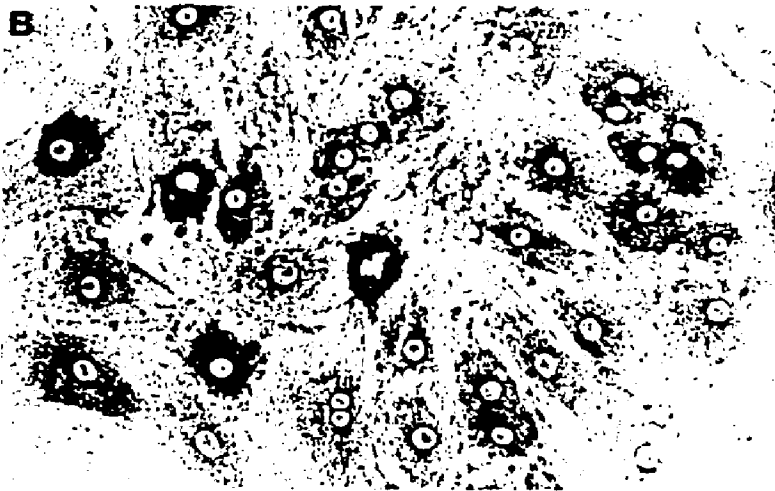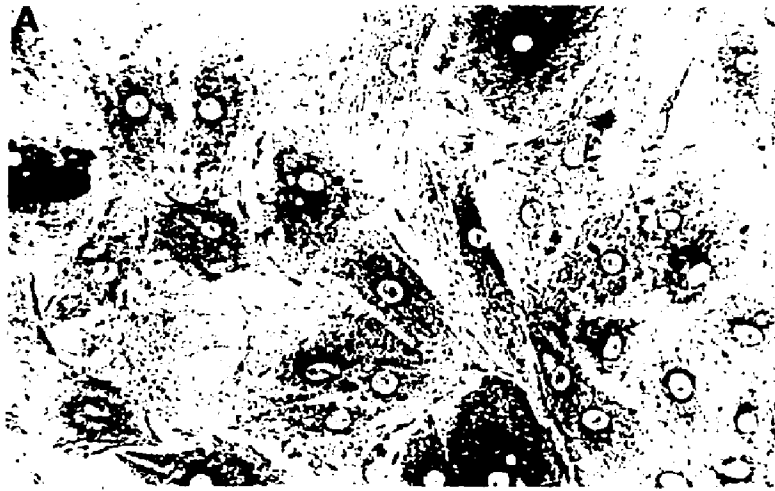
Figure 6.

Negative control (day 8) for non-specific 3β-HSD staining for A. control, B. LH (10 ng/ml) and C. PGF$_{2\alpha}$ (10 ng/ml) treatments (×150).
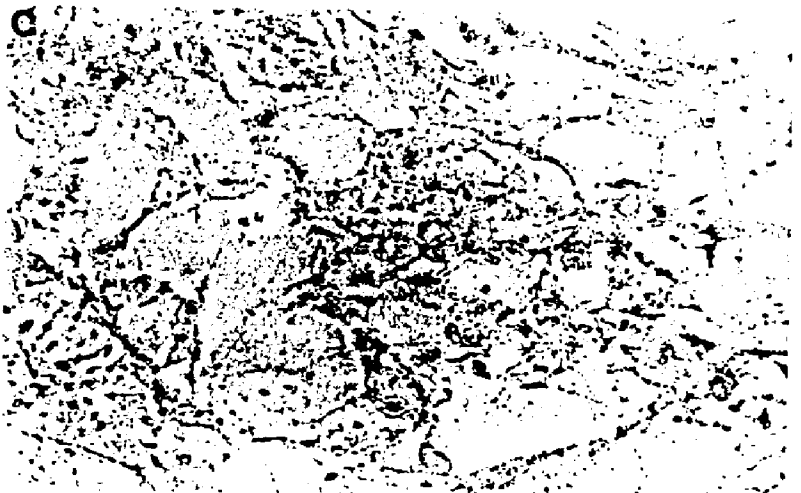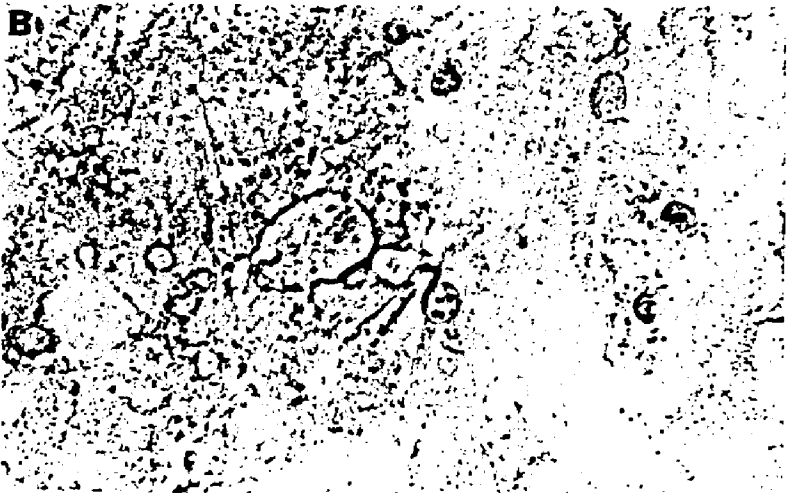
Figure 7.

Percent 3β-HSD-positive cells of total cells during an 8 day culture period for control, LH (10 ng/ml) and $PGF_{2\alpha}$ (10 ng/ml) treatments. Bars with different superscripts are statistically different (P<0.05). mean±SEM, n=3.

FIGURE 7

Figure 8.
Effect of A23187 (10 nM) and EGTA (0.3 mM) on day 2 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml) and LH + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different. mean±SEM, n=7.

FIGURE 8
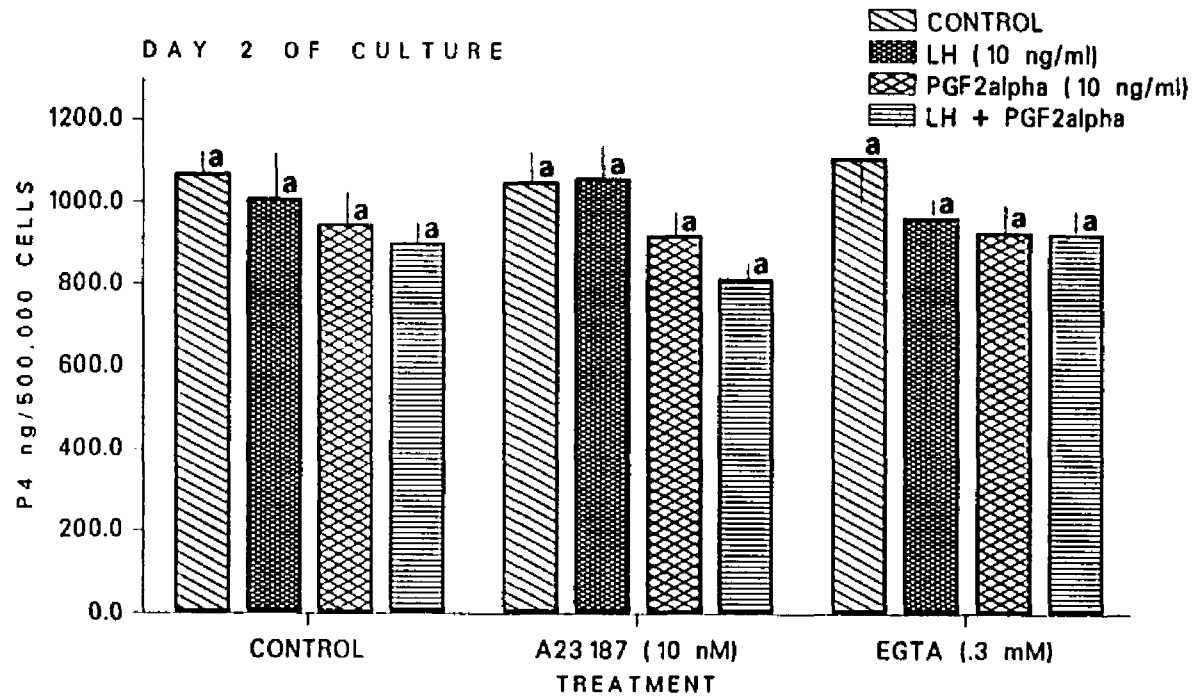
Figure 9.
      Effect of A23187 (10 nM) and EGTA (0.3 mM) on day 4 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml) and LH + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different (P<0.05). mean±SEM, n=7.

FIGURE 9

DAY 4 OF CULTURE

CONTROL
LH (10 ng/ml)
PGF2alpha (10 ng/ml)
LH + PGF2alpha

64
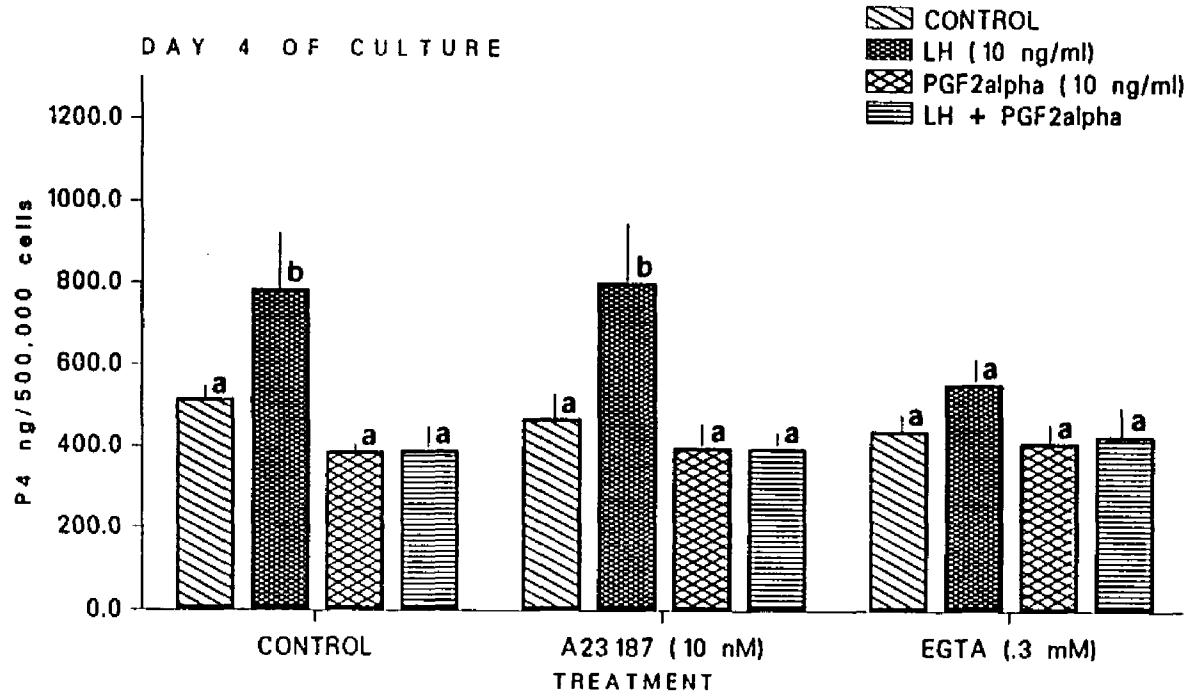
Figure 10.
Effect of A23187 (10 nM) and EGTA (0.3 mM) on day 6 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml) and LH + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different (P<0.01); LH vs. A23187+LH (P<0.05). mean±SEM, n=7.

FIGURE 10

DAY 6 OF CULTURE

CONTROL
LH (10 ng/ml)
PGF2alpha (10 ng/ml)
LH + PGF2alpha

Figure 11.
     Effect of A23187 (10 nM) and EGTA (0.3 mM) on day 8 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml) and LH + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different (P<0.01). LH vs. A23187+LH (P<0.05). mean±SEM, n=7.

FIGURE 11
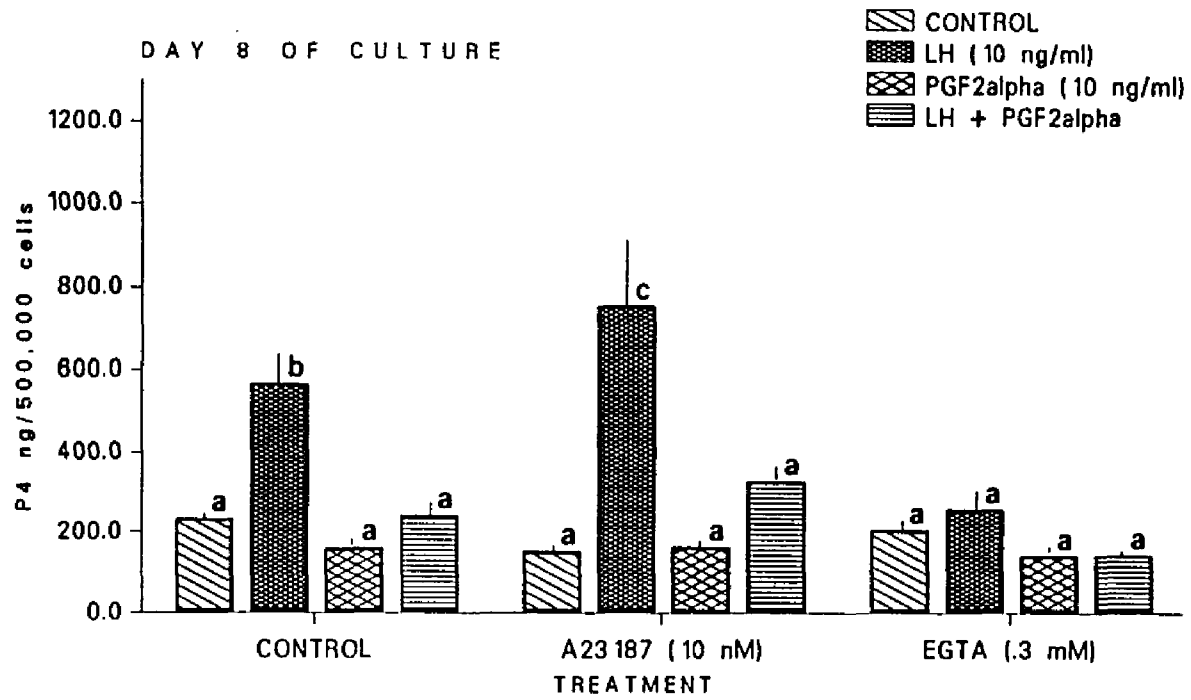
Figure 12.
PGF$_{2\alpha}$ production by day 2 cultured bovine luteal cells treated with LH (10 ng/ml), A23187 (10 nM), A23187 + LH, EGTA (0.3 mM) and EGTA + LH. Bars with different superscripts are statistically different. mean±SEM, n=7.

FIGURE 12
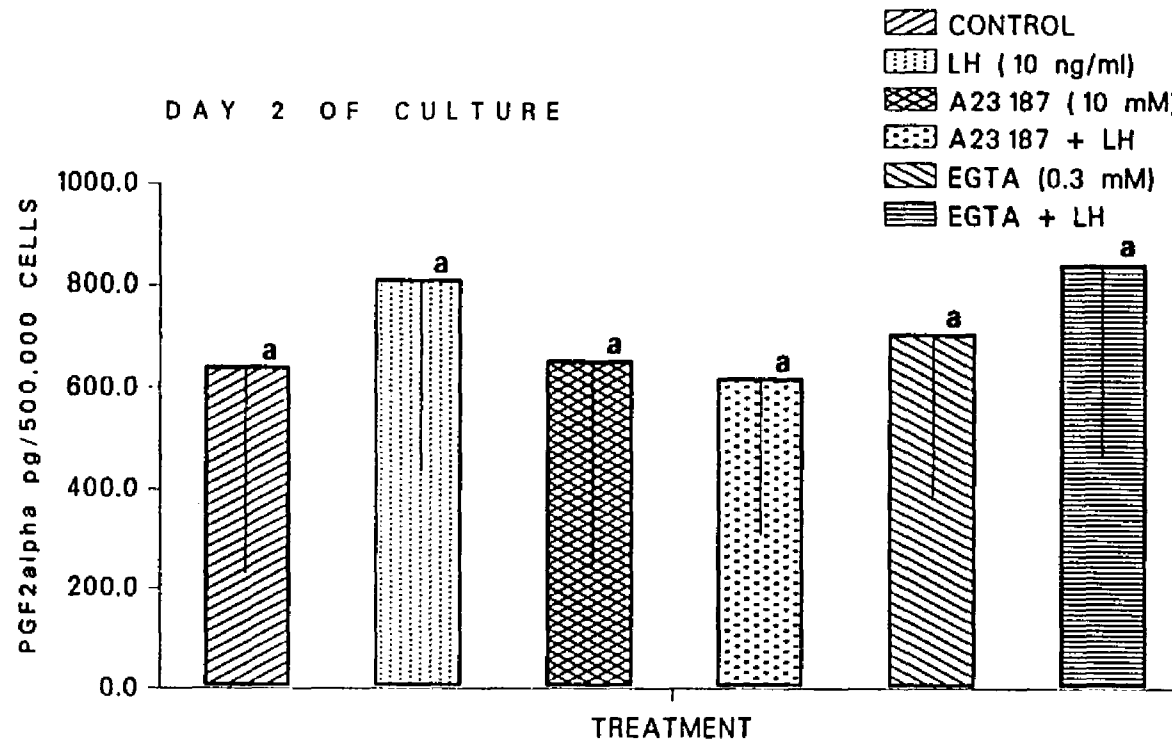
Figure 13.

PGF$_{2\alpha}$ production by day 4 cultured bovine luteal cells treated with LH (10 ng/ml), A23187 (10 nM), A23187 + LH, EGTA (0.3 mM) and EGTA + LH. Bars with different superscripts are statistically different. mean±SEM, n=7.

FIGURE 13

DAY 4 OF CULTURE

Legend:
- CONTROL
- LH (10 ng/ml)
- A23 187 (10 nM)
- A23 187 + LH
- EGTA (0.3 mM)
- EGTA + LH

Y-axis: PGF2alpha pg/500,000 CELLS (0.0, 200.0, 400.0, 600.0, 800.0, 1000.0)

X-axis: TREATMENT
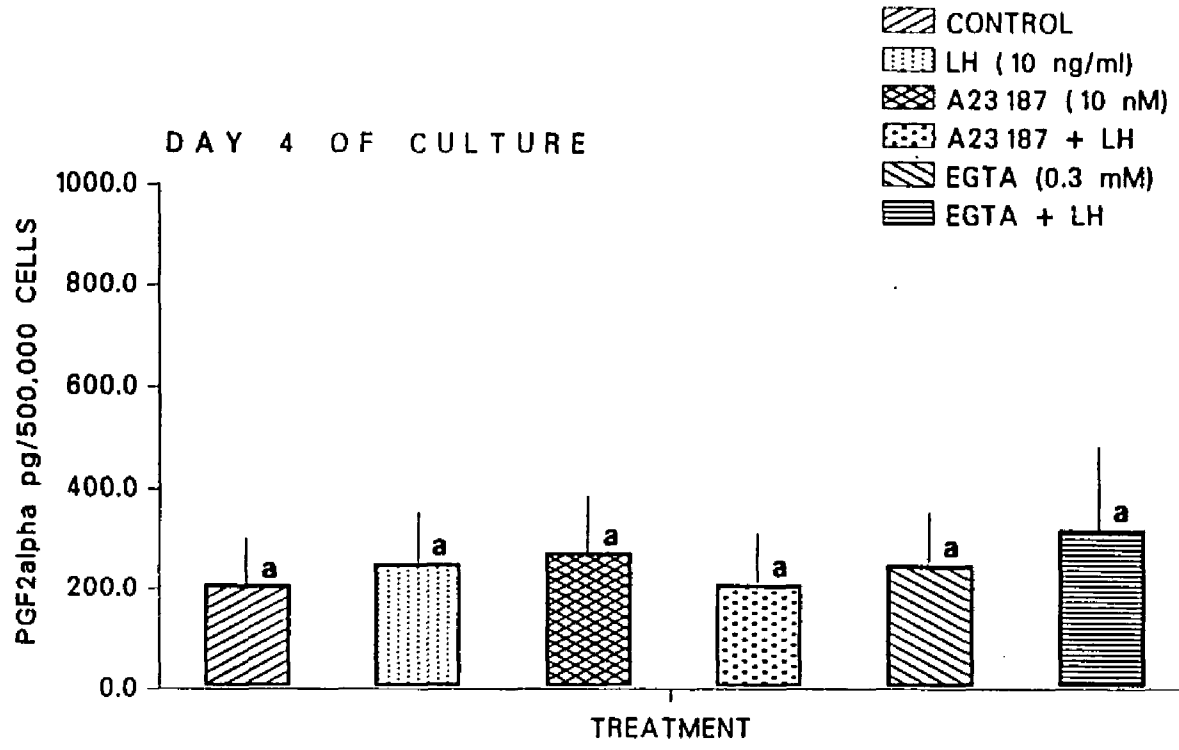
Figure 14.
      Effect of TMB-8 (2 μM), CCCP (50 μM), TFP (1 μM) and W-7 (5 μM) on day 2 P_4 production by bovine luteal cells in control, LH (10 ng/ml) and $PGF_{2\alpha}$ (10 ng/ml) treatments. Bars with different superscripts are statistically different. mean$\pm$SEM, n=4.

FIGURE 14

DAY 2 OF CULTURE

Legend:
- CONTROL
- LH (10 ng/ml)
- PGF2alpha (10 ng/ml)

Y-axis: P4 ng/500,000 cells (0.0, 200.0, 400.0, 600.0, 800.0, 1000.0, 1200.0)

X-axis (TREATMENT): CONTROL, TMB-8, CCCP, TFP, W-7
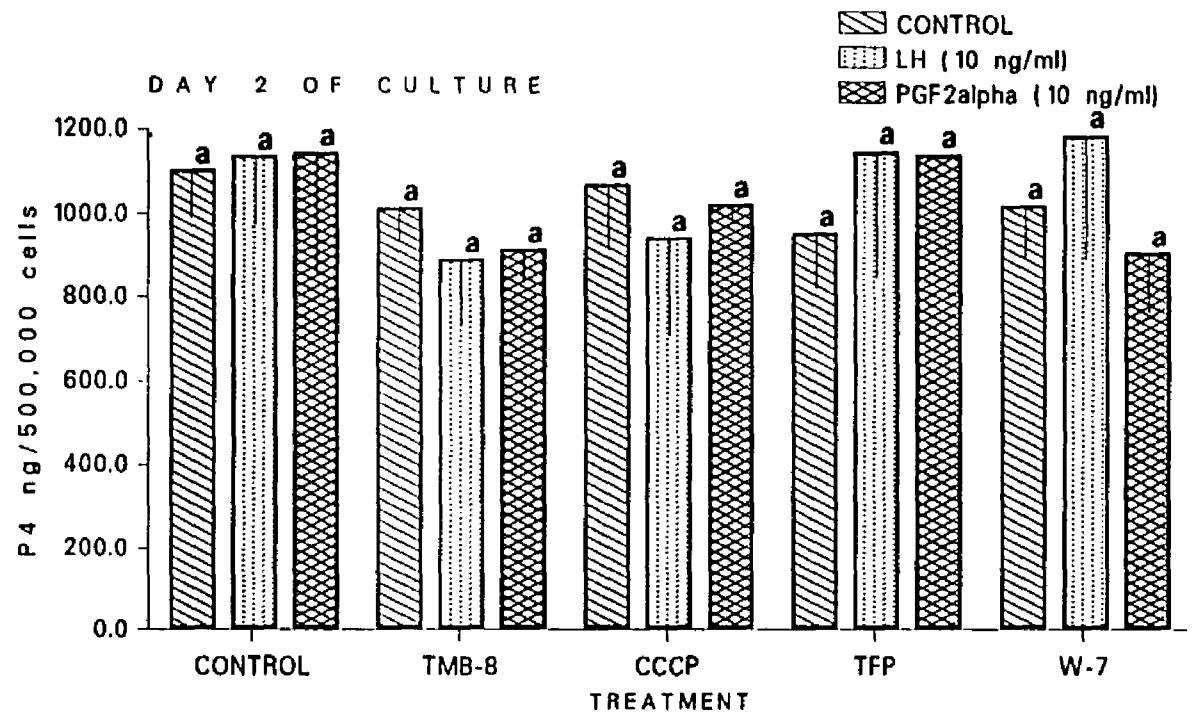
Figure 15.

    Effect of TMB-8 (2 µM), CCCP (50 µM), TFP (1 µM) and W-7 (5 µM) on day 4 P_4 production by bovine luteal cells in control, LH (10 ng/ml) and $PGF_{2\alpha}$ (10 ng/ml) treatments. Bars with different superscripts are statistically different (P<0.01). mean±SEM, n=4.
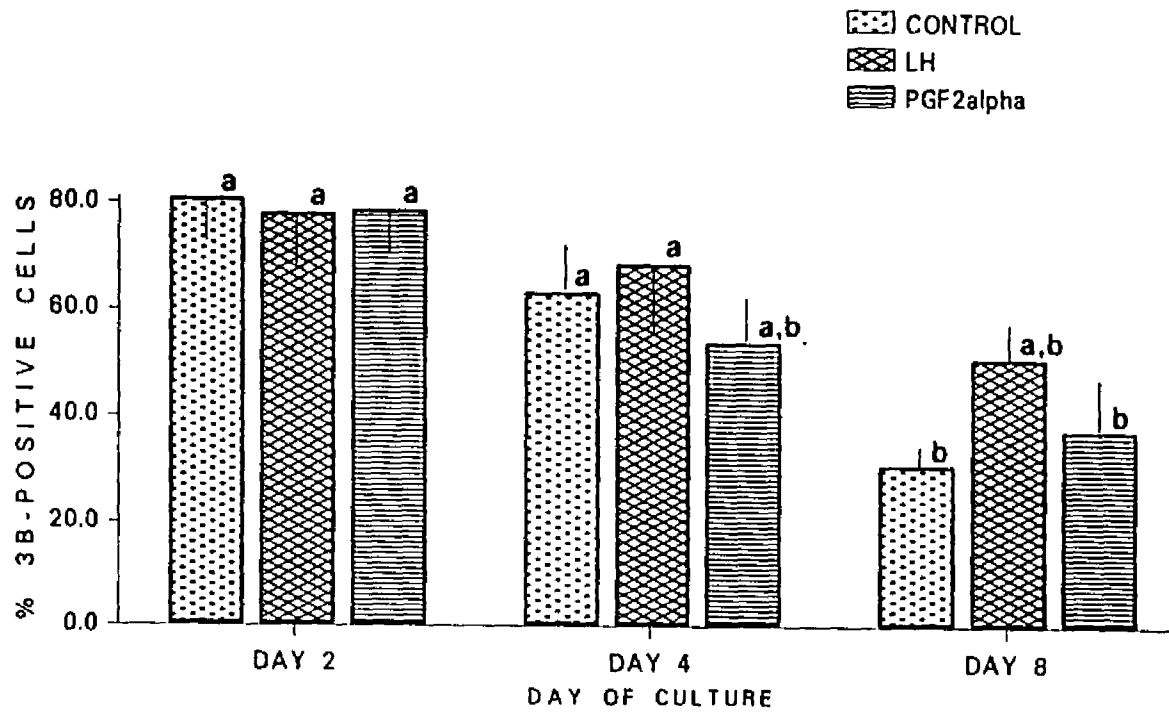
FIGURE 15

DAY 4 OF CULTURE

Legend:
- CONTROL
- LH (10 ng/ml)
- PGF2alpha (10 ng/ml)

Figure 16.

Effect of TMB-8 (2 μM), CCCP (50 μM), TFP (1 μM) and W-7 (5 μM) on day 6 P_4 production by bovine luteal cells in control, LH (10 ng/ml) and $PGF_{2\alpha}$ (10 ng/ml) treatments. Bars with different superscripts are statistically different (P<0.01); TFP+LH and W-7+LH (P<0.05). mean±SEM, n=4.

FIGURE 16

DAY 6 OF CULTURE

Legend:
- ▨ CONTROL
- ▦ LH (10 ng/ml)
- ▩ PGF2alpha (10 ng/ml)

Y-axis: P4 ng/500,000 cells (0.0, 200.0, 400.0, 600.0, 800.0, 1000.0, 1200.0, 1400.0)

X-axis (TREATMENT): CONTROL, TMB-8, CCCP, TFP, W-7
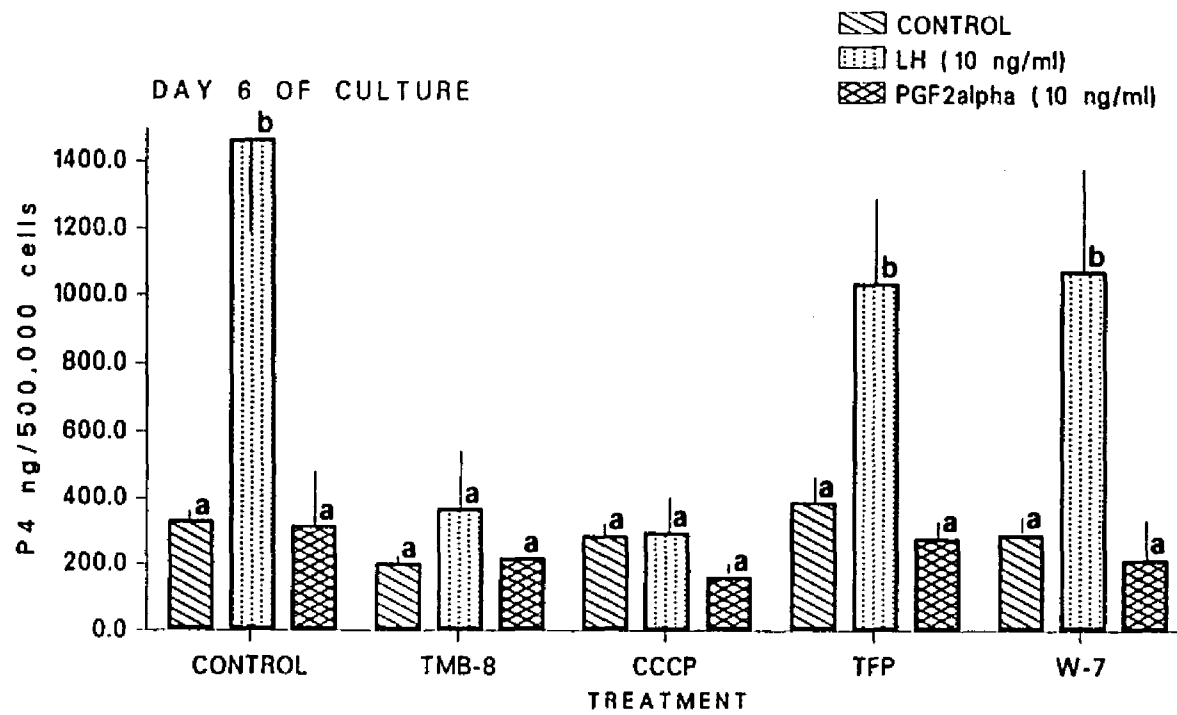
Figure 17.
Effect of TMB-8 (2 μM), CCCP (50 μM), TFP (1 μM) and W-7 (5 μM) on day 8 P_4 production by bovine luteal cells in control, LH (10 ng/ml) and $PGF_{2\alpha}$ (10 ng/ml) treatments. Bars with different superscripts are statistically different (P<0.05); LH (P<0.01). mean±SEM, n=4.

FIGURE 17

DAY 8 OF CULTURE

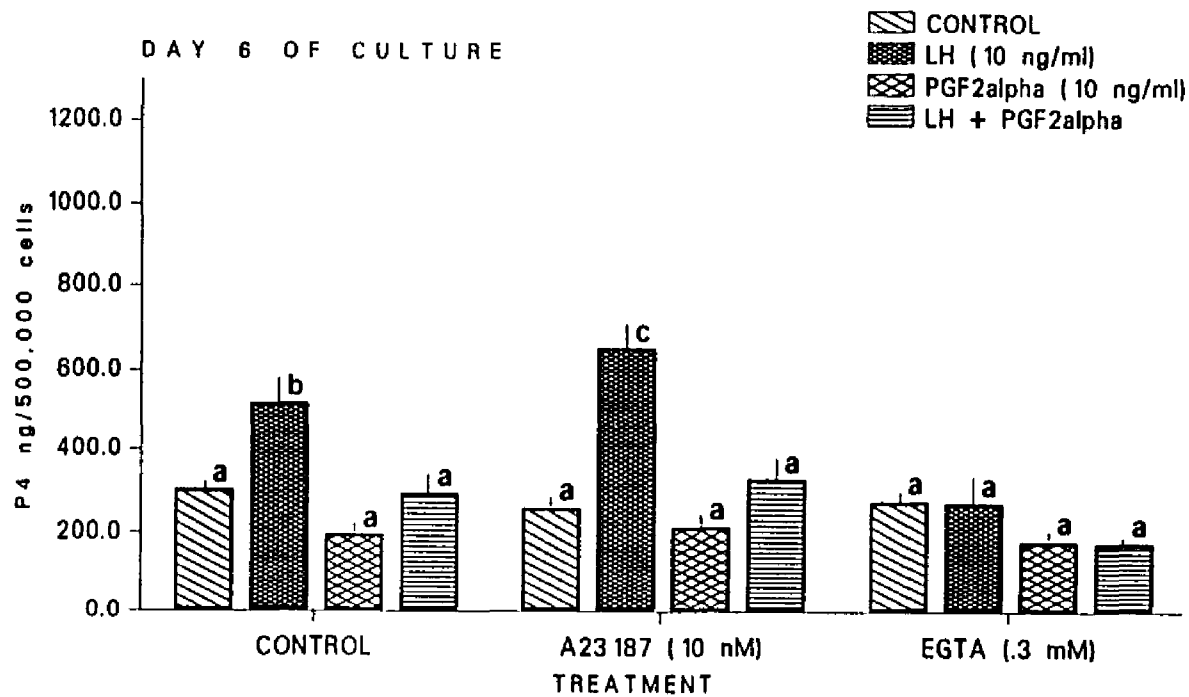NN CONTROL
LH (10 ng/ml)
PGF2alpha (10 ng/ml)

Figure 18.

Effect of phenol-containing and phenol-free Ham's F12 culture medium on day 2 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different. mean±SEM, n=5.

FIGURE 18

DAY 2 OF CULTURE

Legend:
- CONTROL
- LH (10 ng/ml)
- PGF2alpha (10 ng/ml)
- E2 (1 ng/ml)
- E2 + LH
- E2 + PGF2alpha

Figure 19.

Effect of phenol-containing and phenol-free Ham's F12 culture medium on day 4 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different (P<0.01). mean±SEM, n=5.

FIGURE 19

DAY 4 OF CULTURE

Legend:
- CONTROL
- LH (10 ng/ml)
- PGF2alpha (10 ng/ml)
- E2 (1 ng/ml)
- E2 + LH
- E2 + PGF2alpha

Y-axis: P4 ng/500,000 cells

X-axis: CULTURE MEDIUM (Ham's F 12, Phenol-Free Ham'sF 12)

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Figure 20.

Effect of phenol-containing and phenol-free Ham's F12 culture medium on day 6 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different ($P<0.05$). mean±SEM, n=5.

FIGURE 20

DAY 6 OF CULTURE

Legend:
- ▦ CONTROL
- ▨ LH (10 ng/ml)
- ▦ PGF2alpha (10 ng/ml)
- ▦ E2 (1 ng/ml)
- ▦ E2 + LH
- ▦ E2 + PGF2alpha

Figure 21.

Effect of phenol-containing and phenol-free Ham's F12 culture medium on day 8 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different (P<0.01). mean±SEM, n=5.

FIGURE 21

DAY 8 OF CULTURE

Legend:
- ▦ CONTROL
- ▨ LH (10 ng/ml)
- ▦ PGF2alpha (10 ng/ml)
- ▤ E2 (1 ng/ml)
- ▩ E2 + LH
- ▦ E2 + PGF2alpha

P4 ng/500,000 cells — CULTURE MEDIUM: Ham's F 12, Phenol-Free Ham'sF 12

Figure 22.

Effect of phenol-containing and phenol-free Ham's F12 culture medium on day 10 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different ($P<0.05$). mean±SEM, n=5.
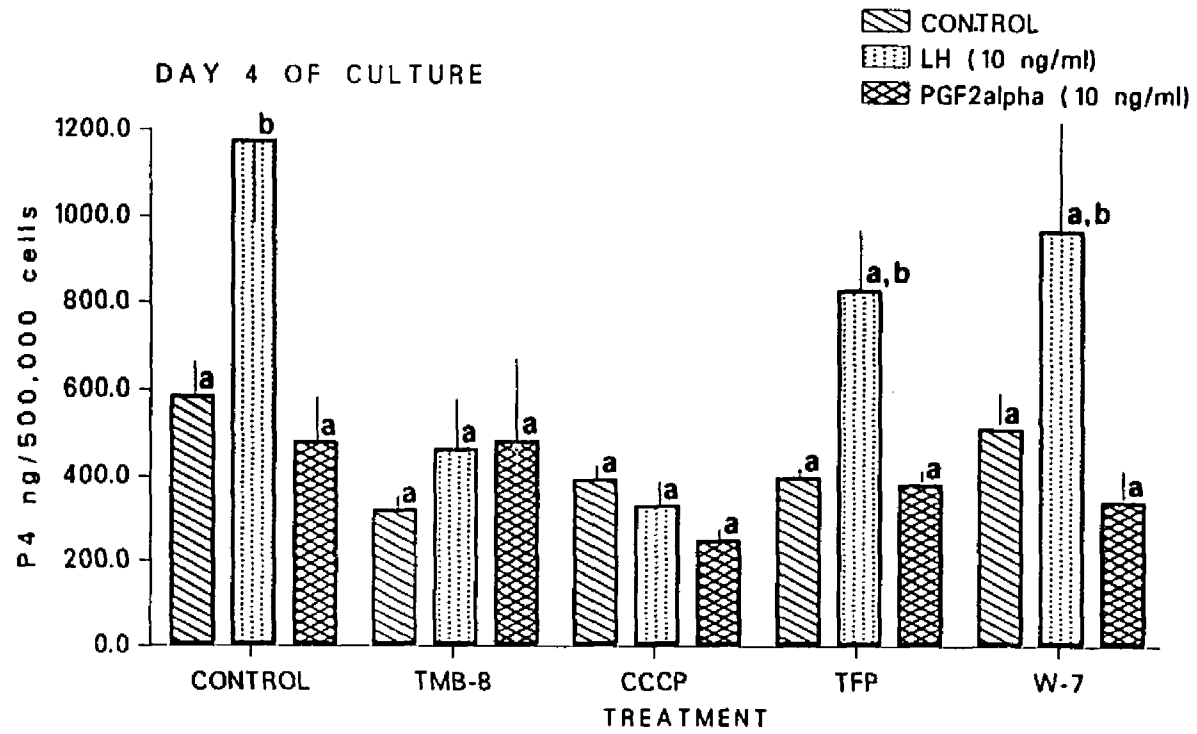
FIGURE 22

DAY 10 OF CULTURE

Legend:
- CONTROL
- LH (10 ng/ml)
- PGF2alpha (10 ng/ml)
- E2 (1 ng/ml)
- E2 + LH
- E2 + PGF2alpha

Y-axis: P4 ng/500,000 cells (0.0, 500.0, 1000.0, 1500.0, 2000.0, 2500.0)

X-axis: CULTURE MEDIUM (Ham's F 12, Phenol-Free Ham'sF 12)
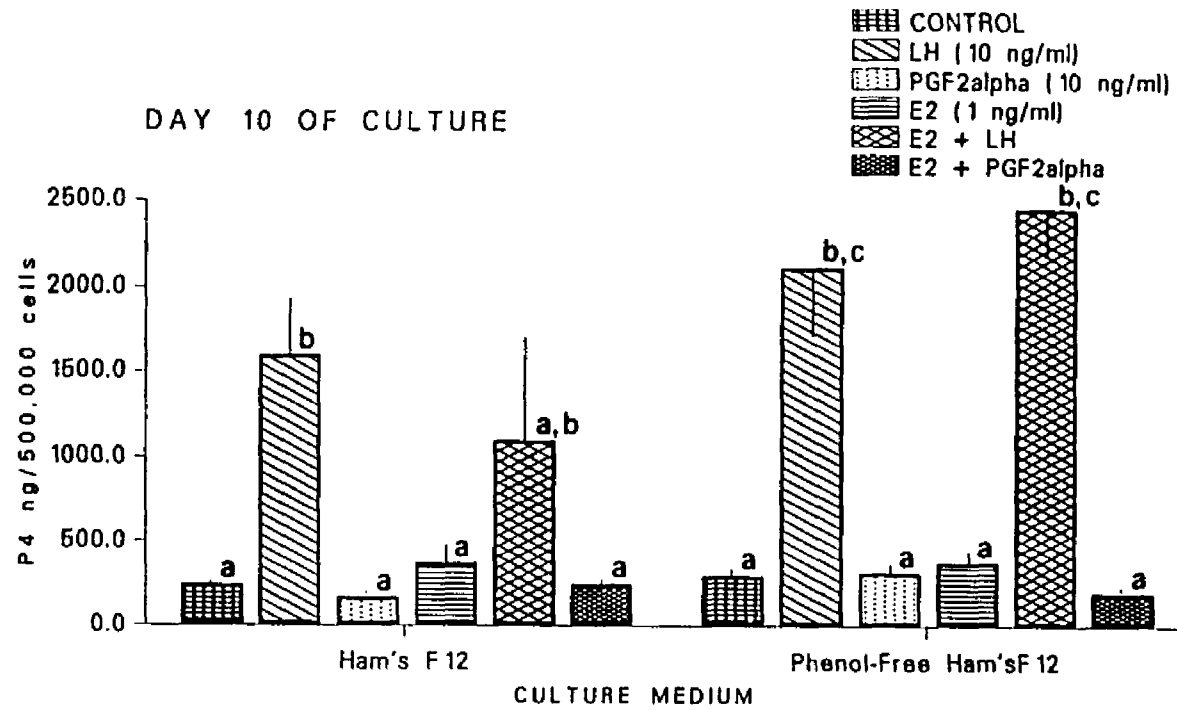
Figure 23.
Effect of phenol-containing and phenol-free MEM culture medium on day 2 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different (P<0.05). mean±SEM, n=2.

FIGURE 23

- CONTROL
- LH (10 ng/ml)
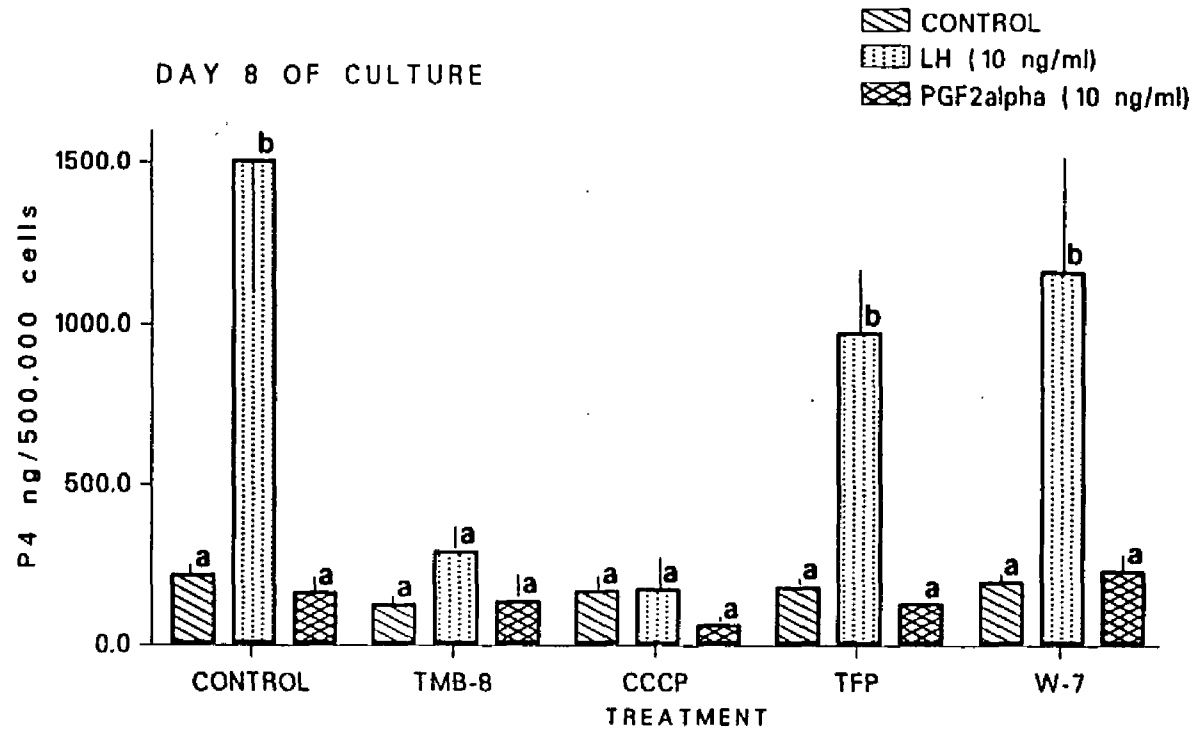- PGF2alpha (10 ng/ml)
- E2 (1 ng/ml)
- E2 + LH
- E2 + PGF2alpha
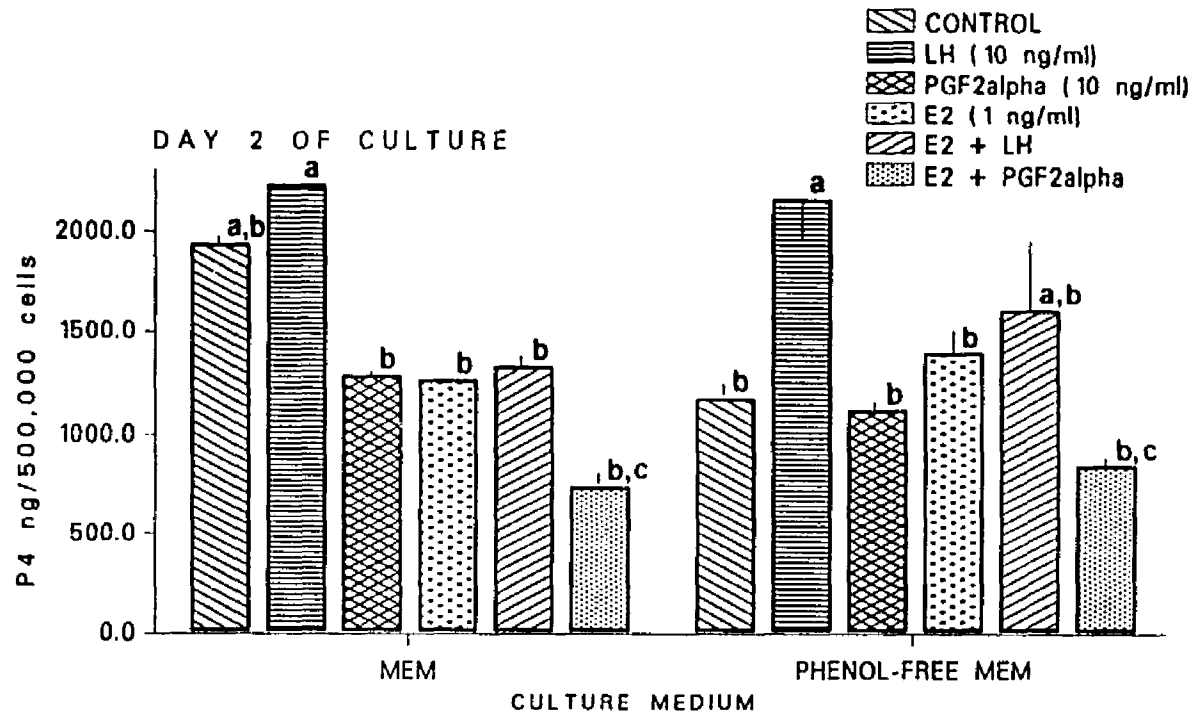
Figure 24.

Effect of phenol-containing and phenol-free MEM culture medium on day 4 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different (P<0.01). mean±SEM, n=2.

FIGURE 24

Legend:
- CONTROL
- LH (10 ng/ml)
- PGF2alpha (10 ng/ml)
- E2 (1 ng/ml)
- E2 + LH
- E2 + PGF2alpha

DAY 4 OF CULTURE

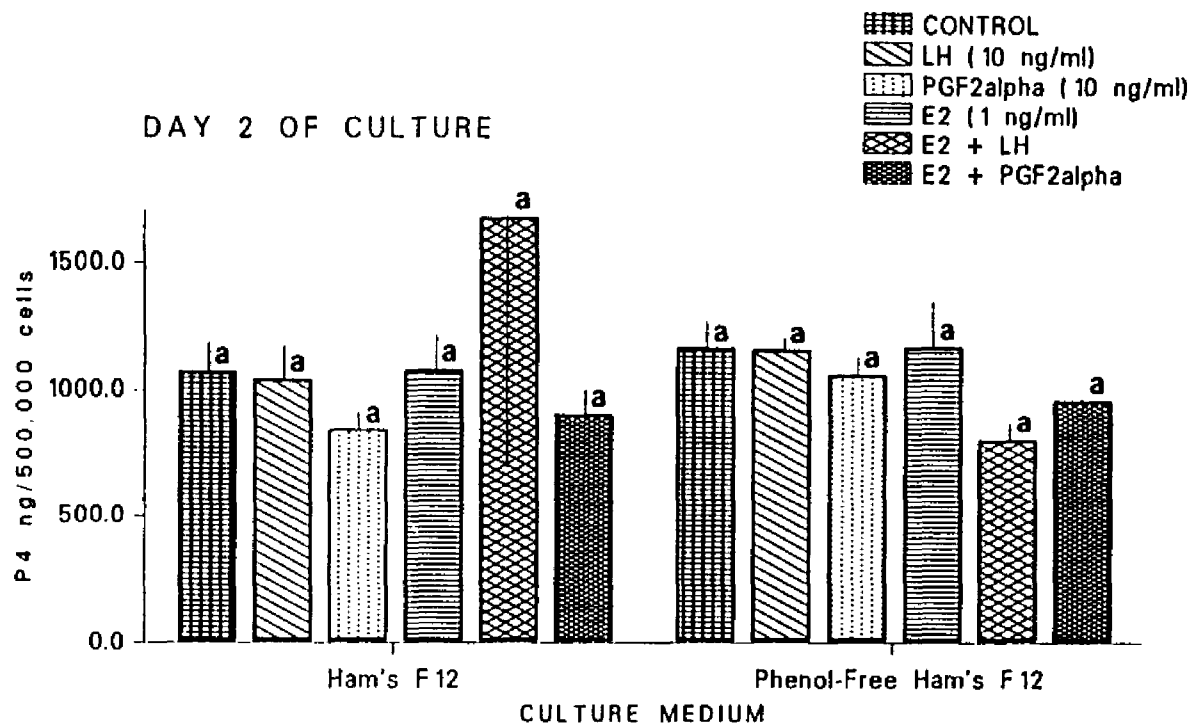P4 ng/500.000 cells

CULTURE MEDIUM
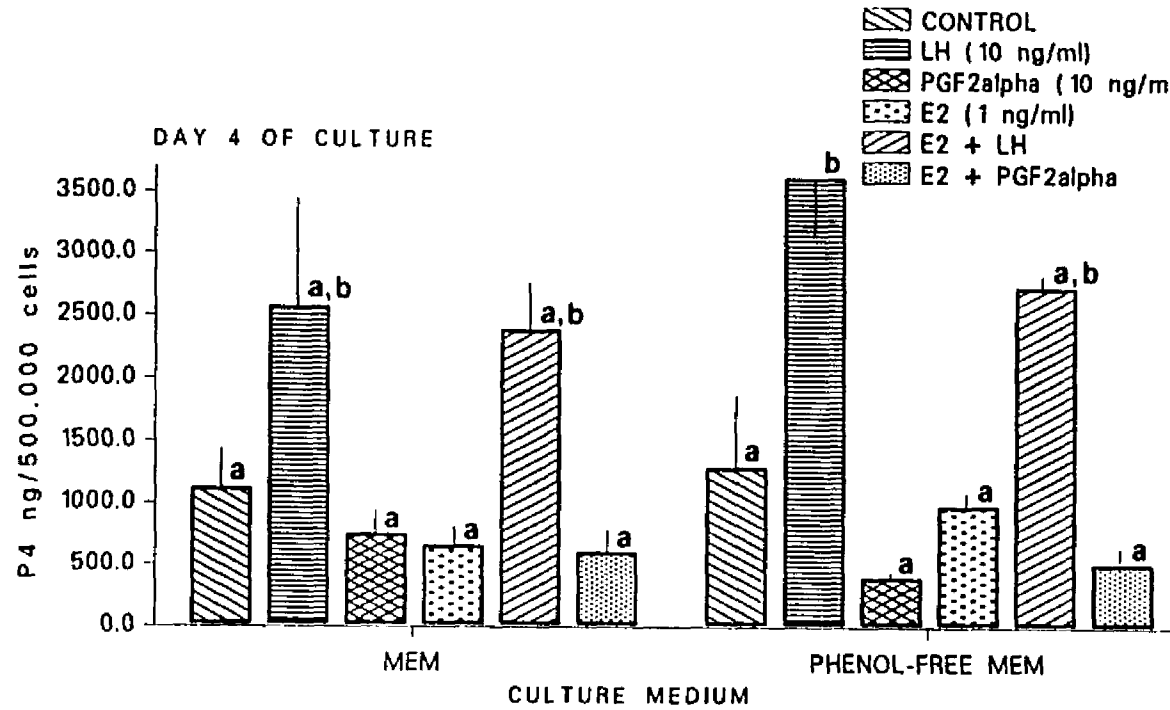
MEM   PHENOL-FREE MEM

Figure 25.

Effect of phenol-containing and phenol-free MEM culture medium on day 6 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different super-scripts are statistically different (P<0.01); E2+LH (phenol-containing and phenol-free MEM) (P<0.05). mean±SEM, n=2.

FIGURE 25

Legend:
- CONTROL
- LH (10 ng/ml)
- PGF2alpha (10 ng/ml)
- E2 (1 ng/ml)
- E2 + LH
- E2 + PGF2alpha

DAY 6 OF CULTURE

P4 ng/500,000 cells

8000.0
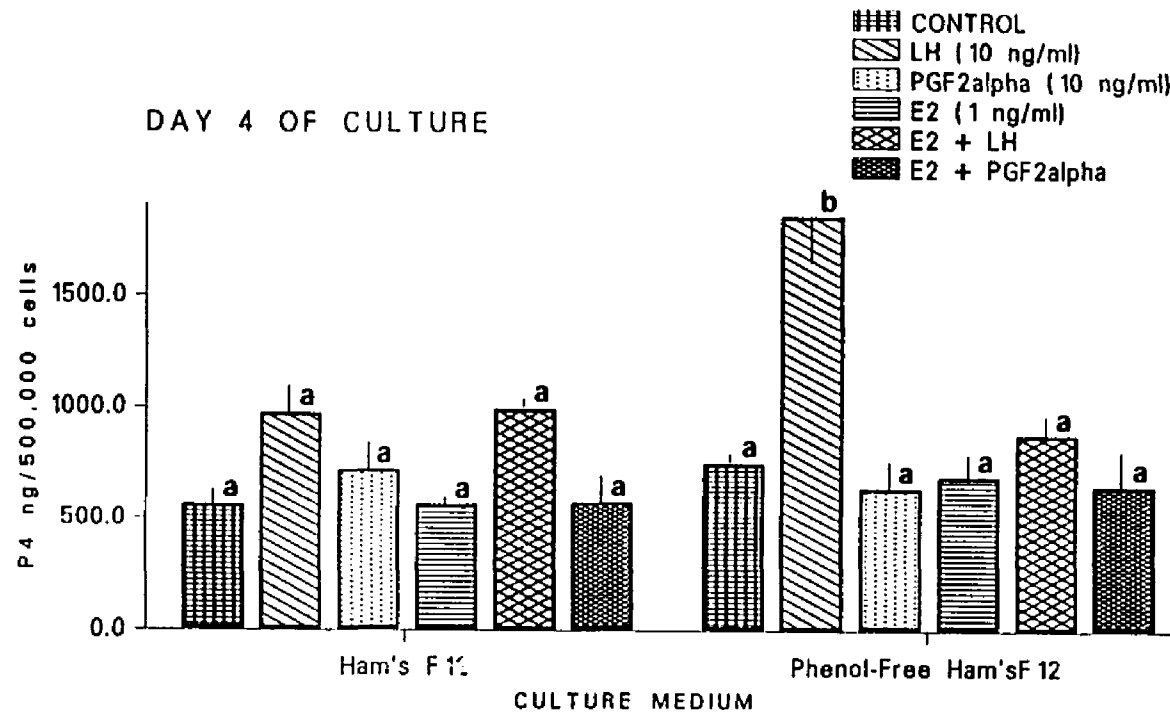6000.0
4000.0
2000.0
0.0

MEM

PHENOL-FREE MEM
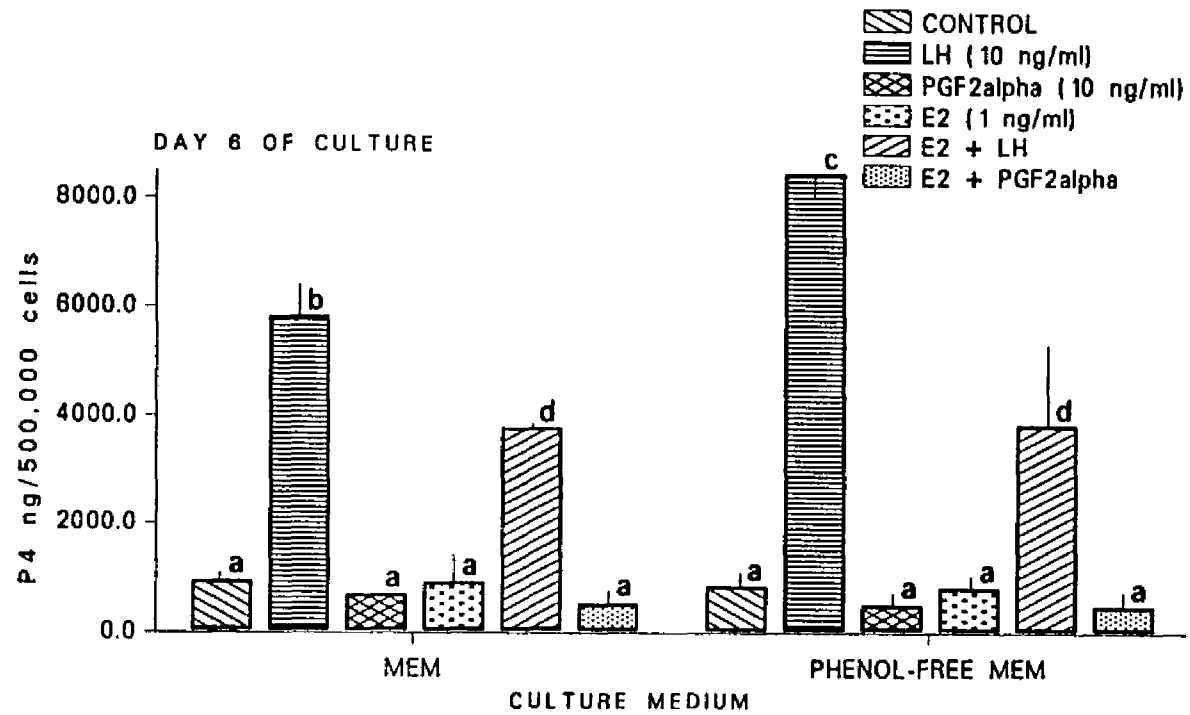
CULTURE MEDIUM

Figure 26.

Effect of phenol-containing and phenol-free MEM culture medium on day 8 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different super-scripts are statistically different (P<0.01); E2+LH (phenol-containing and phenol-free MEM) (P<0.05). mean±SEM, n=2.

FIGURE 26

Legend:
- ⊠ CONTROL
- ☰ LH (10 ng/ml)
- ⊠ PGF2alpha (10 ng/ml)
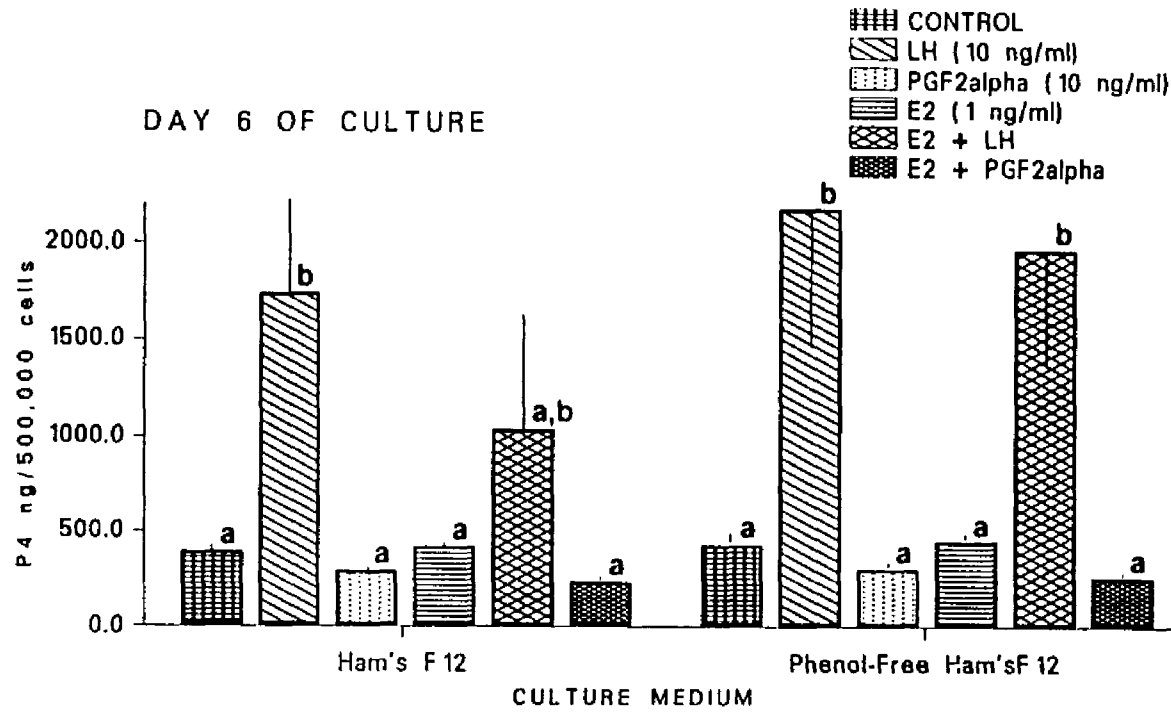- ⊡ E2 (1 ng/ml)
- ⊘ E2 + LH
- ▦ E2 + PGF2alpha
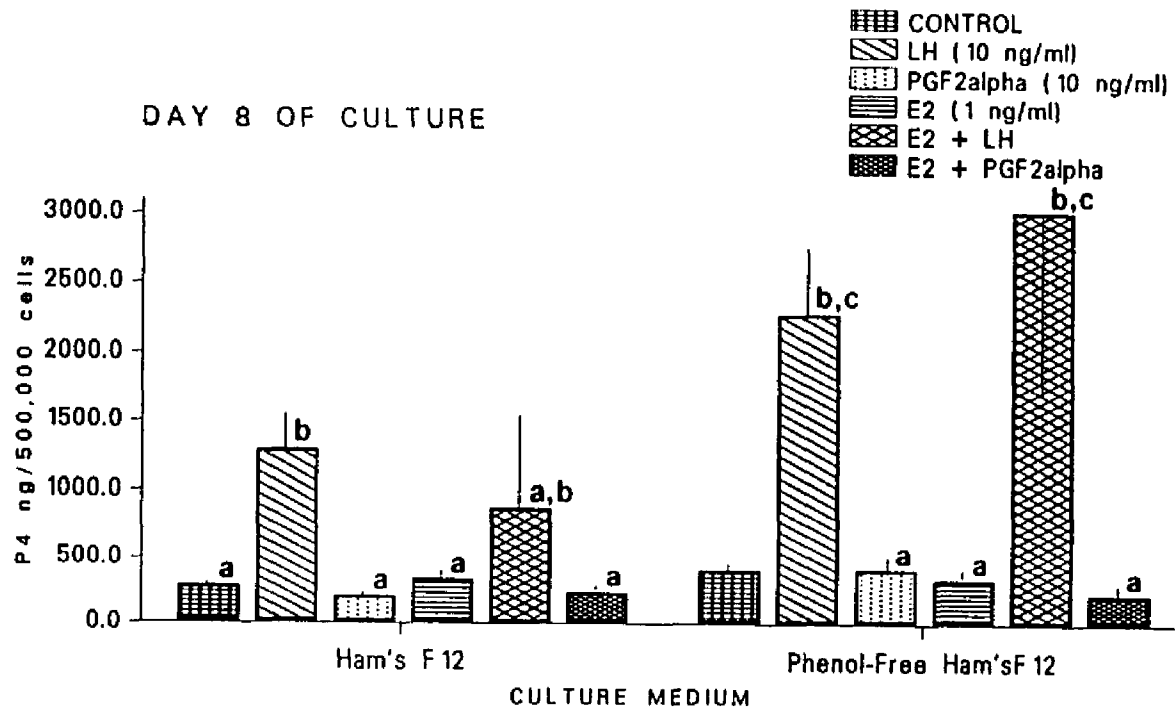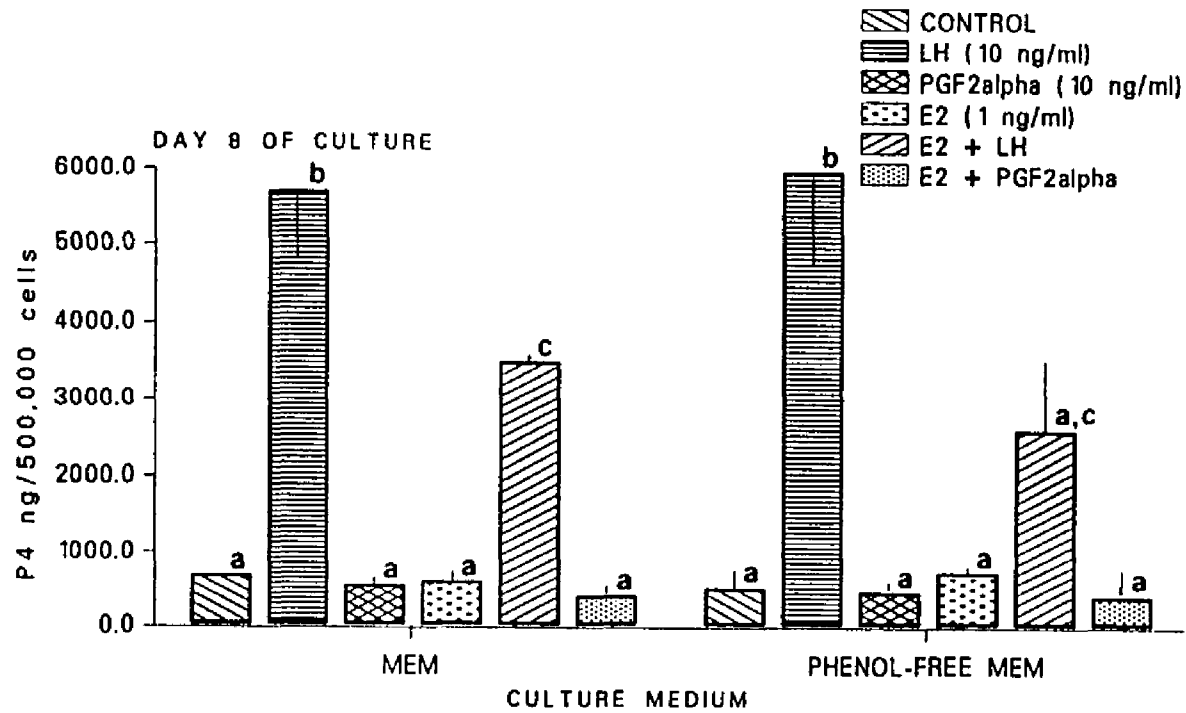
Figure 27.

Effect of phenol-containing and phenol-free MEM culture medium on day 10 P_4 production by bovine luteal cells in control, LH (10 ng/ml), $PGF_{2\alpha}$ (10 ng/ml), E_2 (1 ng/ml), E_2 + LH and E_2 + $PGF_{2\alpha}$ treatments. Bars with different superscripts are statistically different (P<0.01); E2+LH (phenol-containing and phenol-free MEM) (P<0.05). mean±SEM, n=2.

FIGURE 27

Legend:
- CONTROL
- LH (10 ng/ml)
- PGF2alpha (10 ng/ml)
- E2 (1 ng/ml)
- E2 + LH
- E2 + PGF2alpha

DAY 10 OF CULTURE

P4 ng/500.000 cells

5000.0
4000.0
3000.0
2000.0
1000.0
0.0

MEM   PHENOL-FREE MEM

CULTURE MEDIUM
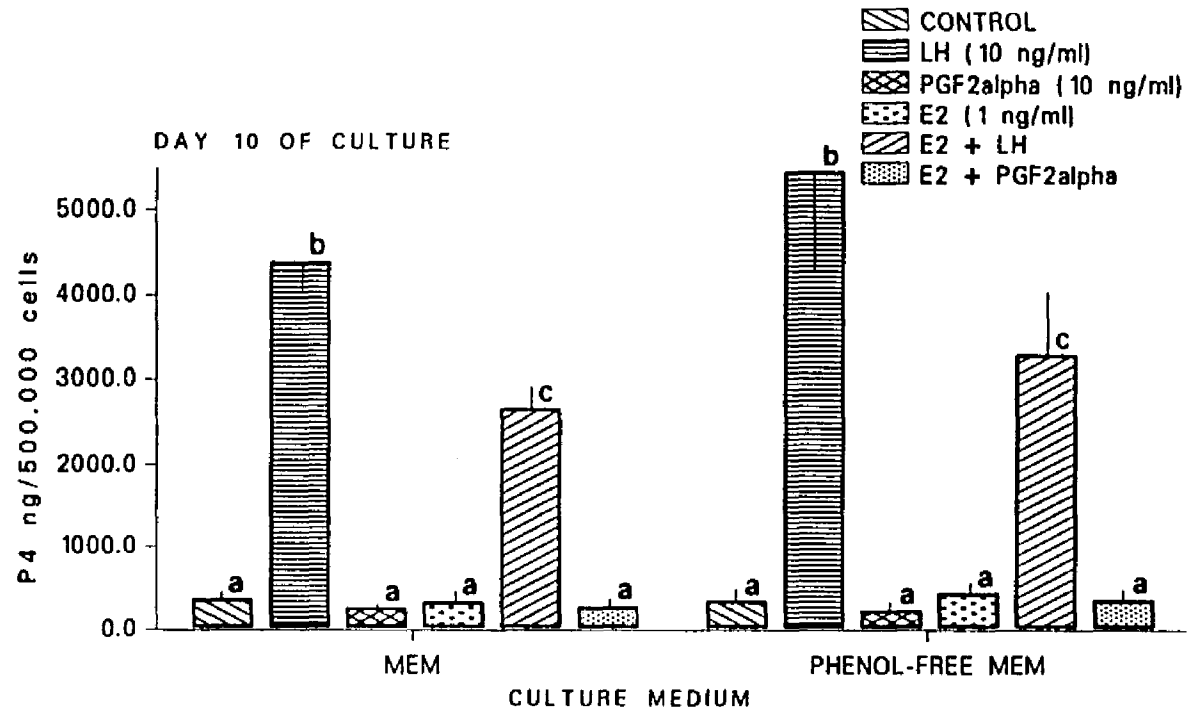
Table 1. Progesterone production by cultured bovine luteal cells
in the presence of Ca^{++} antagonist TMB[a]

	n	Day 2	Day 4	Day 6	Day 8
			P_4^b		
CONTROL	4	1106.3±118.2	587.3±88.2	331.3±24.4	224.3±22.3
TMB-8					
100 μM	2	2793.6±926.9	447.7±68.5	ND	ND
200 μM	2	952.6±43.9	365.7±14.8	279.1±52.4	ND
2 μM	4	1015.2±86.1	321.3±38.2	202.5±16.1	133.0±24.8

[a]3,4,5-trimethoxy-benzoic acid 8-(diethylamino) octyl ester
[b]progesterone ng/500,000 cells; mean±SEM

Table 2. Progesterone production by cultured bovine luteal cells
in the presence of Ca^{++} antagonist CCCP[a]

	n	Day 2	Day 4	Day 6	Day 8
			P_4^b		
CONTROL	4	1106.3±118.2	587.3±88.2	331.3±24.4	224.3±22.3
CCCP					
500 μM	2	2960.9±1590.2	659.6±283.9	551.5±208.6	194.3±177.8[c]
50 μM	4	1073.5±183.8	394.2±40.1	288.1±35.8	175.0±36.2
5 μM	2	1574.9±368.9	728.3±199.9	410.2±31.5	240.1±10.9

[a]carbonyl cyanide m-chlorophenyl-hydrazone
[b]progesterone ng/500,000 cells; mean±SEM
[c]mean±SD

Table 3. Progesterone production by cultured bovine luteal cells
in the presence of calmodulin antagonist TFP[a]

	n	P_4^b			
		Day 2	Day 4	Day 6	Day 8
CONTROL	4	1106.3±118.2	587.3±88.2	331.3±24.4	224.3±22.3
TFP					
1 μM	4	953.1±136.4	398.6±27.1	389.0±90.4	186.8±17.9
100 nM	2	1159.1±37.8	835.4±302.1	406.4±39.5	245.0±9.2
10 nM	2	1088.9±152.5	572.0±38.6	491.4±68.2	294.4±60.4

[a]trifluoperazine
[b]progesterone ng/500,000 cells; mean±SEM

Table 4. Progesterone production by cultured bovine luteal cells
in the presence of calmodulin antagonist W-7[a]

	n	P_4^b			
		Day 2	Day 4	Day 6	Day 8
CONTROL	4	1106.3±118.2	587.3±88.2	331.3±24.4	224.3±22.3
W-7					
50 μM	2	1387.9±285.5	603.7±28.6[c]	419.2±41.2[c]	201.7±4.7[c]
5 μM	4	1022.3±131.8	514.2±84.9	292.4±50.3	205.0±13.8
0.1 μM	2	1323.7±40.3	547.5±6.7	363.2±116.4	187.4±20.2

[a]N-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide
[b]progesterone ng/500,000 cells; mean±SEM
[c]mean±SD

Table 5. Progesterone production by cultured bovine luteal cells in the presence of a voltage-sensative Ca^{++} channel blocker, NF^a

	n	P^b_4			
		Day 2	Day 4	Day 6	Day 8
CONTROL	2	842.0±114.2	477.1±138.2	416.3±153.9	340.0±114.1
NF					
1 µM	2	1167.6±113.7	628.5±48.9	443.4±16.8	368.1±8.3
100 nM	2	2168.3±1279.1	806.1±489.0	373.6±82.5	281.2±69.8
10 nM	2	1908.7±106.9	663.6±12.0	479.4±56.1	319.0±60.8

[a]nifedipine
[b]progesterone ng/500,000 cells; mean±SEM

DISCUSSION

Since $PGF_{2\alpha}$ was demonstrated to be a luteolysin in 1970 (McCracken et al., 1970), researchers have been trying to elucidate its definitive role in luteolysis. Conflicting *in vivo* and *in vitro* results have hampered researchers from discerning the true mechanism of action of $PGF_{2\alpha}$ in bovine luteal regression. The use of a long-term culture system to evaluate luteal function incorporates features of short-term *in vitro* experiments over time. Results from these controlled, long-term *in vitro* experiments may be more characteristic of *in vivo* situations.

Experiment I

As in previous long-term cultures of bovine luteal cells, LH treatment was ineffective in elevating P_4 levels early in the culture (day 2). This phenomenon has been noted routinely (Pate and Condon, 1984) and may be attributed to transient effects induced by a protease contaminant in the collagenase used during the dissociation procedure (Ascoli and Segaloff, 1986). Later days of culture showed the characteristic stimulation of P_4 by LH in a serum-free cell culture system. The initial effect of $PGF_{2\alpha}$ in bovine luteal cell culture was to stimulate P_4 production as is seen in short term incubations (Speroff and Ramwell, 1970; Hixon and Hansel, 1979). Subsequent days in culture demonstrate $PGF_{2\alpha}$ as slightly inhibitory or ineffective in altering basal P_4 production, while completely inhibiting LH-stimulated P_4 synthesis. This is in agreement with other long-term studies investigating the action of $PGF_{2\alpha}$ *in vitro* (Pate and Condon, 1984).

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The present study utilized 6-well culture plates in which approximately 2×10^5 cells per well attached yielding a plating efficiency of 80%. This number of cells was maintained in this system through the 4th day of culture. As the culture progressed, total numbers of cells were decreased approximately 20% in each treatment group. This loss in cell numbers also corresponded with a loss in numbers of steroidogenically active cells. Initial cultures reflected 80% steroidogenically active cells. This population was assumed to be comprised of both small and large cells, although numbers of each could not be determined. Other cell types such as fibroblasts from the intact luteal tissue were maintained in culture and, along with unstained luteal cells, represented the steroidogenically inactive population. By day 8, control cultures demonstrated an approximate 50% loss in the steroidogenic cell population. Similar losses were noted in $PGF_{2\alpha}$-treated wells and cannot be attributed to the treatment. This inability of $PGF_{2\alpha}$ to alter steroidogenic enzymes correlates with its inability to significantly alter basal P_4 levels in culture. The lack of $PGF_{2\alpha}$ effect on 3β-HSD activity is not in agreement with previous *in vivo* studies in which $PGF_{2\alpha}$-induced significant decreases in rat ovarian tissue (Dwyer and Church, 1979) and ovine luteal tissue (McClellan et al., 1977). In both of these studies, actual enzyme activity was measured whereas, the present study utilized only enzyme localization as an indicator of enzyme activity and may have lead to inconclusive results. Additional studies incorporating enzyme activity measurements must be performed to ascertain a definitive effect of $PGF_{2\alpha}$ on 3β-HSD in the bovine luteal cell.

LH stimulated a greater maintenance of steroidogenic cells than was seen in control cultures on day 8. Another study using cultured bovine luteal cells demonstrated similar results (Fairchild and Pate, 1987). The present study supports the work of Poff et al.(1987) which

showed that luteal cells initially cultured in the absence of LH lost the ability to respond to trophic hormones later in the culture.

Therefore, bovine luteal cells in culture appear to require some baseline luteotropic support to maintain their steroidogenic potential. $PGF_{2\alpha}$, in this system, does not have any influence on steroidogenic activity of 3β-HSD although additional investigations are necessary to substantiate these results.

Experiment II

Several researchers have demonstrated the influence of Ca^{++} and various Ca^{++} environments on steroidogenesis of luteal cells in short term experiments (Higuchi et al., 1976; Behrman etal., 1979; Davis et al., 1987a,b). To this date, the long-term effects of Ca^{++} in vitro have not been investigated for bovine luteal cells.

The first set of studies in this experiment indicated an influence of Ca^{++}-enrichment and Ca^{++}-depletion on bovine luteal steroidogenesis over an 8 day culture period. By day 4 of culture, it was evident that Ca^{++}-depletion was detrimental to LH-stimulation of P_4 but had no effect on basal P_4 production. EGTA is known to chelate Ca^{++} selectively over other divalent cations such as Mg^{++} (Lefkowitz et al., 1970) and bind Ca^{++} on a per mole basis. Therefore, EGTA at 0.3 mM was expected to completely chelate the 0.3 mM extracellular Ca^{++} provided by the culture medium. This effect of Ca^{++}-depletion on LH function was evident throughout the remainder of the culture and agrees with previous short-term data (Higuchi et al., 1976; Farese and Prudente, 1978 and Davies et al., 1985). The inability of Ca^{++}-depletion to effect basal P_4 was noted in these first two studies while, Davies et al. (1985) did show reduced basal

cortisol synthesis in adrenocortical cells in the presence of EGTA. Lahav et al. (1983) demonstrated a decrease in LH generated cAMP in rat luteal cells cultured for 90 minutes in the presence of EGTA, but this was found to be insignificant. This study utilized pooled tissue from several rats and the large inherent variability between corpora lutea of different animals may be responsible for the error in the system yielding insignificant results.

The influence of the Ca^{++}-enriched environment, induced by the Ca^{++} specific ionophore A23187, on LH-stimulated P_4 was not apparent until later in the culture. Basal P_4 appeared uneffected by ionophore treatment throughout the culture. The effect of A23187 on LH-stimulated P_4 was first noted on day 6, when the presence of A23187 stimulated additional increases in P_4 production by LH above those in LH-only treated flasks. Similar results were noted in ovine luteal cells incubated *in vitro* (Sawyer et al., 1979).

The beneficial influence of Ca^{++} on LH and cAMP-stimulated steroidogenesis has been noted in previous studies (Sawyer et al., 1979; Veldhuis et al., 1984). Studies have shown that protein hormone stimulation of steroidogenesis not only requires Ca^{++} but also increases intracellular Ca^{++} in many target cell types such as: ACTH stimulation of pregnenolone in the adrenal (Farese and Prudente, 1978), LH stimulation of P_4 in granulosa cells (Asem et al., 1987b), LH stimulation of testosterone in Leydig cells (Sullivan and Cooke, 1986) and hCG and LH stimulation of P_4 production in bovine luteal cells (Davis et al., 1986 and Davis et al., 1987a).

Treatment with $PGF_{2\alpha}$ resulted in decreases of basal P_4 throughout the culture which were not significantly different from controls in any treatment group. These results are in agreement with a previous study from this laboratory (Pate and Condon, 1984). $PGF_{2\alpha}$ in this study was able to completely suppress LH-stimulated P_4 production from day 4 of culture to its

conclusion on day 8. This suppression was also seen in previous long-term bovine luteal cultures (Pate and Condon, 1984). In the present study the absence of available Ca^{++} from the extracellular environment did not alter the $PGF_{2\alpha}$-induced suppression of LH action. Similar effects have been seen in cultures of rat luteal cells (Lahav et al., 1983). In short-term cultures of bovine luteal cells, $PGF_{2\alpha}$-induced increases in intracellular Ca^{++} which were independent of extracellular Ca^{++} (Davis et al., 1987b). This inhibition of LH-stimulated P_4 may be due to $PGF_{2\alpha}$-induced Ca^{++} stimulation, and the activation of pathways other than those mediated by cAMP. These results suggest that $PGF_{2\alpha}$ may be mediating its effects via an alternative second messenger system, possibly through phosphoinositides and protein kinase C (PK-C). Recent work by Davis et al. (1987b) showed that $PGF_{2\alpha}$ caused rapid increases in all inositol phosphates, primarily IP_3 and a transient increase in intracellular Ca^{++} which reached its maximum at 30 seconds and remained elevated for 10 minutes.

It has been implied that $PGF_{2\alpha}$ works through a Ca^{++}-dependent PK-C in bovine luteal cells (Benhaim et al., 1987). An active PK-C mechanism has been identified in these cells (Davis and Clark, 1983). Studies using 4 h rat Leydig cell cultures showed an initial increase in androgen production in response to the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA) (Moger, 1985). TPA directly activates the PK-C enzyme without mobilizing Ca^{++}. Twenty-four hours later TPA induced non-significant decreases in basal androgen levels and significant inhibition of LH-stimulated androgen production. $PGF_{2\alpha}$ in luteal cells appears to act as a phorbol ester by initially stimulating P_4 production and subsequently reducing basal levels insignificantly, while completely inhibiting LH-stimulated P_4 accumulation. Another study by Papadopoulos et al. (1985) demonstrated that phorbol ester-stimulated PK-C blocked

cAMP accumulation in rat Leydig cells, suggesting that PK-C exerts a negative feedback on cAMP-signalling systems. Possibly, $PGF_{2\alpha}$-stimulation of PK-C is exerting a negative feedback inhibition of adenylate cyclase and cAMP-stimulation in bovine luteal cells.

PK-C has also been suggested to downregulate insulin and transferrin receptors (review: Nishizuka, 1986). Previous work done in this lab indicated that maintenance of LH-stimulated steroidogenesis in cultured luteal cells requires insulin and transferrin (Poff et al., 1988). Possibly, $PGF_{2\alpha}$-stimulated PK-C reduces LH-stimulated P_4 production by limiting the availability of insulin and transferrin.

$PGF_{2\alpha}$ action on LH-stimulated P_4 was also uneffected by Ca^{++}-enrichment on days 4 through 8, although slight increases in P_4 were seen in the LH + $PGF_{2\alpha}$ + A23187 treatment on days 6 and 8. A study using rat luteal cells suggested that $PGF_{2\alpha}$ functions by increasing intracellular Ca^{++}, resulting in a blockade of adenylate cyclase activity (Baum and Rosberg, 1987). These researchers showed inhibition of LH-induced cAMP and cAMP-stimulation of P_4 in the presence of A23187, resembling that of $PGF_{2\alpha}$. Presumably, the ionophore elevated Ca^{++} and acted as a mimic of $PGF_{2\alpha}$. It appears that the relative level of intracellular Ca^{++} determines its stimulatory or inhibitory action (Lefkowitz et al., 1970). Slight increases in Ca^{++} would be beneficial to steroidogenesis while levels above a critical threshold (approximately 10^{-4} M) would be inhibitory. It is proposed that A23187 at the 10 nM dose used in this study was only capable of increasing Ca^{++} such that it was stimulatory to LH activation of P_4. Baum and Rosberg (1987) utilized 10 μM A23187 and noted inhibition of steroidogenesis presumably due to excessive Ca^{++}. Preliminary short-term (2h) incubations of luteal cells with A23187 (1 μM) yielded a 40% inhibition of P_4 as compared to control values (data not

shown).

Bovine luteal cells cultured in the presence of nifedipine (NF) showed no alteration of basal or LH-stimulated P_4 production. This data suggests that voltage sensitive Ca^{++} channels may not have a role in steroidogenesis by bovine luteal cells and is supported by similar conclusions in the rat (Gore and Behrman, 1984). Other studies suggest the presence of voltage sensitive Ca^{++} channels in ovine (Higuchi et al., 1976) and porcine species (Veldhuis and Klase, 1982).

Luteal production of endogenous $PGF_{2\alpha}$ was unaltered by Ca^{++} depletion or Ca^{++} enrichment. These results were not supported by research in other cell types which suggest that EGTA decreased $PGF_{2\alpha}$ output (Riley and Poyser, 1987) while, A23187 caused increased production of all prostaglandins, including $PGF_{2\alpha}$ (Silvia and Homanics, 1987; Zijlstra et al., 1987). Both of these studies utilized a higher dose of ionophore (10 µM), which may account for the ineffectiveness of A23187 at 10 nM in the present work. LH produced slight increases of $PGF_{2\alpha}$ in day 2 and 4 of culture. These increases were not significant and agree with previous results from bovine long-term cultures (Pate, 1987). Grazul et al. (1987) was able to demonstrate significant stimulation of $PGF_{2\alpha}$ by LH in short-term cultures of late midcycle bovine luteal cells. The significance of these results is not known at this time, but LH-induced increases of $PGF_{2\alpha}$ in late cycle corpora luteal may function in an autocrine manner to prepare the tissue for regression.

Two Ca^{++}-antagonists, TMB-8 and CCCP, as well as two calmodulin antagonists, TFP and W-7, were employed to further investigate the effects of Ca^{++} in bovine luteal steroidogenesis. The high doses of TMB-8 (100 µM) and CCCP (500 µM) appeared to induce

significant P_4 production (Table 1 and 2). These values were artificially elevated because of significant cell loss (approximately 60%) due to pharmacological action of the treatment. These values must, therefore, be disregarded. The dosages of TMB-8 (2 μM), CCCP (50 μM), TFP (1 μM) and W-7 (5 μM) were selected on the basis of preliminary trials shown in Tables 1-4, and values sited in the literature.

As in the EGTA cultures, Ca^{++} antagonists completely inhibited LH-stimulated P_4 accumulation. Similar results on steroid production using TMB-8 were seen in adrenal glomerulosa cells (Kojima et al., 1985). Levels of P_4 in $PGF_{2\alpha}$-treated flasks were unaltered by the presence of Ca^{++} antagonists. Inhibitors of calmodulin, TFP and W-7, suppressed LH-stimulation of P_4 only slightly, suggesting that calmodulin-bound Ca^{++} does not play as major a role in P_4 steroidogenesis as does free Ca^{++}. Similar results were seen using these drugs in rat luteal cultures (Lahav et al., 1987). This does not agree with studies showing that inhibition of calmodulin did impact on steroid production in bovine adrenal (Davies et al., 1985) and avian granulosa cells (Asem and Hertelendy, 1987a).

This experiment strongly supports the role of free, intracellular Ca^{++} in the regulation of bovine luteal steroidogenesis. LH-induced P_4 production is dependent on a free Ca^{++} environment, but is only somewhat modified by the Ca^{++} binding protein calmodulin. The action of $PGF_{2\alpha}$ in mediating a reduction of LH-stimulated P_4 production cannot be determined in a Ca^{++}-free environment since LH-stimulation itself requires Ca^{++}. Other studies have suggested that $PGF_{2\alpha}$ may be working through a phospholipid-Ca^{++}-dependent protein kinase and this may be the case in this system.

Experiment III

As early as 1966, E_2 was implicated in the regression process in the cow (Wilbank, 1966). A cooperative effect of E_2 + $PGF_{2\alpha}$ has been demonstrated in cattle (Hixon and Hansel, 1979). This study showed that E_2 + $PGF_{2\alpha}$ (7 mg) could induce regression in cycling animals, while $PGF_{2\alpha}$ (7 mg) alone was ineffective.

The site of E_2 binding in target cells remains unclear although recent evidence suggests E_2 binds directly to nuclear receptors to initiate physiological responses (review: Walters, 1985). The presence of E_2 binding proteins has been identified in bovine corpora lutea (Kimball and Hansel, 1974). It has become apparent that E_2 has a direct action on the corpus luteum in a number of species including the cow.

The present study showed a direct inhibitory effect of E_2 (1 ng/ml) on LH-stimulated P_4 synthesis in long-term cultures of bovine luteal cells. A direct inhibitory effect of E_2 has been demonstrated in rat Leydig cells (Hsueh et al., 1978), porcine luteal cells (Grazul et al., 1986) and bovine luteal cells (Williams and Marsh, 1978). This is in contrast to another study utilizing incubations of bovine luteal cells (Hixon and Hansel, 1979). This short-term study used doses of E_2 comparable to the present experiment, but could not show an effect of E_2 on basal or LH-stimulated P_4. The present study used an estrogen glucuronide which increases the solubility of E_2 in aqueous solutions, which may explain its ability to act in this system as opposed to that of Hixon and Hansel.

E_2-producing follicles are present on the bovine ovary on most days of the cycle (Matton et al., 1981; Ireland et al., 1984). The potential exists for pituitary LH pulses late in the cycle

to stimulate follicular E_2 production leading to reduced LH-response at the level of the CL. This is supported by data indicating that exogenous E_2 decreases LH-stimulated adenylate cyclase activation by 52% in rabbit luteal cells (Kirchick and Birnbaumer, 1983). These researchers suggested that E_2 decreases the N-component of adenylate cyclase as well as actual LH receptor numbers. Possibly, E_2 acts in a similar manner in bovine luteal cells.

E_2 in this experiment was unable to alter basal P_4 in phenol-containing or -free Ham's F12 medium. This agrees with short-term studies with bovine luteal cells which showed the same effect (Hixon et al., 1983). The combined treatment of E_2 + $PGF_{2\alpha}$ could not significantly alter basal P_4 production, suggesting that the possible cooperative effect of these two agents is via an indirect pathway. This is in agreement with Hixon and Hansel (1979) who could not demonstrate an additive effect with E_2 + $PGF_{2\alpha}$ in short-term bovine cell cultures.

The influence of phenol red, a weak estrogen, on bovine luteal steroidogenesis was demonstrated in this experiment. Phenol red is present in most commercial preparations of cell culture medium in doses (1 µg/ml to 10 µg/ml) exceeding or comparable to levels of hormone treatments used in the present experiments. The possible impact of phenol red has been investigated in other cell types (Hofland et al., 1987; Sheen and Katzenellenbogen, 1987), but never in cultured bovine luteal cells.

Phenol red levels in Ham's F12 culture medium (1 µg/ml) do effect LH-stimulated steroidogenesis. Maximum stimulation occurs by day 4 in phenol-free medium while LH-stimulated P_4 in phenol-containing medium lags behind and never reachs the same level. Possibly, phenol red acts estrogenically to downregulate LH receptors, which never fully recover

during the 10 day culture period.

Substantial differences between LH-stimulated P_4 in phenol-containing (10 µg/ml) and phenol-free MEM were only statistically evident on day 6, although the trend was maintained throughout the culture. I feel this insufficiency was due to the low n # and would be corrected with more replicates. Absolute levels of basal P_4 and LH-stimulated P_4 were much higher in MEM than in Ham's F12. This effect was independent of phenol red and may be due to the higher concentrations of amino acids, particularly glutamine, in MEM.

Results from this study have indicated an influence of E_2 at the cellular level in the cultured bovine luteal cell, although interactions between E_2 + $PGF_{2\alpha}$ were not evident. Further experimentation utilizing various doses of both E_2 and $PGF_{2\alpha}$ may elucidate a cooperative action. The presence of phenol red in commercial cell culture medium has definite effects on E_2 target cells, and its presence must be considered in evaluating data from *in vitro* studies.

In conclusion, results from this study have further defined our bovine luteal cell culture system. In the first experiment, absolute levels of P_4 remained constant only in the presence of LH. Total cell number was demonstrated to decline slightly throughout the culture in control, LH and $PGF_{2\alpha}$ treatments. Numbers of 3β-HSD positive cells also decreased throughout the culture for control and $PGF_{2\alpha}$ treatments but not for LH. This suggests the need for some baseline luteotropic support to maintain steroidogenesis in cultured bovine luteal cells. The second experiment demonstrated that the LH response is Ca^{++} dependent while the importance of Ca^{++} in the action of $PGF_{2\alpha}$ remains undetermined. The presence of free, intracellular Ca^{++} was shown to be a regulator of luteal steroidogenesis while calmodulin-bound Ca^{++} may not be as

important. In the third experiment, estrogen and phenol red were shown to have a direct inhibitory action on bovine luteal cells. Both E_2 treatment and phenol red decrease the LH-response in cultured bovine luteal cells. The action of $PGF_{2\alpha}$ appeared to be unaltered in the presence of phenol red or E_2. The mechanism of action of $PGF_{2\alpha}$ was partially elucidated and we have provided further knowledge of the functionality of $PGF_{2\alpha}$ in luteal suppression.

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APPENDIX A

LONG TERM DISSOCIATION OF BOVINE LUTEAL CELLS

1. Spin plasma sample in centrifuge for 15 minutes at 2000 rpm. Remove plasma with pasteur pipette and freeze in 3 dram vial.

2. All subsequent procedures must be done in SterilGard™ hood using aseptic techniques and sterile equipment.

3. Remove connective tissue from corpus luteum. Weigh mass in grams.

4. Keep CL in Ham's F-12 w/Hepes medium after washing; keep on ice.

5. Weigh out enough collagenase to equal 2000 units of activity.

$$\frac{(2000\ u/g)\ (g\ of\ CL)}{(units\ activity\ on\ bottle\ in\ mg)} = amt.\ collagenase$$

set aside in weighing paper.

6. Prepare medium w/Bovine Serum Albumin (BSA, 0.5%).

Weigh out: 0.5g/100ml medium

7. Add 7ml BSA-medium to 10ml beaker; keep on ice.

8. Using clean razor blade, forceps & microscope slide, section the CL into 4 quarters.

9. Slice each quarter into strips & then into 1mm cubes.

10. Place cubes into 10ml beaker with medium as each quarter is prepared. Chop finely with scissors.

11. Pour off medium from 10ml beaker; add fresh medium to beaker & wash tissue into dissociation flask.

12. Place dissociation flask in heater-stirrer at 37°C for 15 min. at setting 3.5.

13. Pour off medium, add new medium and collagenase; replace in heater-stirrer at 37°C for 45 min.-1 hour. Using a broken-tip pipet, aspirate cells every 10 min. to aid dissociation.

14. Serum coat flasks with 10% serum for 1-2 hours. Add approximately 3ml/flask. Rinse flasks twice with serum-free medium at the end of 2 hours.

15. Pour off medium into 2-15cc centrifuge tubes.

16. Add collagenase & BSA-medium to remaining tissue and repeat dissociation procedure.

17. Centrifuge cells at 800rpm (150 xg) for 10 min.

18. Wash w/BSA-free medium (Ham's F-12) 3 times at 600rpm (80 xg) for 10 min.

19. Prepare 2 borosilicate tubes with 900μl vital stain (Trypan Blue 1:10) in each; vortex cells, add 100μl of cells to each tube; vortex mixture and quickly load hemacytometer with contents from 1 tube for each side.

20. Count cells in 5 grids on both sides and average to determine cells/grid.

$$\text{cells/grid} \times 10^4 \times \text{dilution factor (10)} = \text{cells/ml}$$

21. Determine desired #cells/flask tube and dilute stock with medium until correct #cells/ml is reached for the # of flasks; add 3ml medium + 1ml of cells to each flask.

22. Prepare treatments to the desired concentration. Add to flasks.

23. Pour off and replace culture medium and treatments in 24 hours. Save and freeze (-20°C) medium for P_4 RIA.

24. Pour off and replace culture medium and treatments every 48 hours for the duration of the culture period.

APPENDIX B

PROGESTERONE RADIOIMMUNOASSAY PROTOCOL

Assay:

1. Make standard progesterone for standard curve. -Defrost 1 vial standard stock progesterone which contains 10 ng/ml. Remove 2 ml and place into a 25 ml glass- stoppered flask. Add 18 ml ethanol (absolute) (a 1:10 dilution); resulting solution will be 1.0 ng/ml. -Amounts for standard curve are: 2.0, 1.0, 0.4, 0.2, 0.1, 0.06, 0.02 ng/ml To obtain these amounts simply pipette 2.0, 1.0, 0.4, 0.2, 0.1, 0.06, 0.02 ml, respectively, into assay tubes. *For each assay there are two standard curves, one before samples and one after the samples; in addition, each point on the curve is done in duplicate, then averaged. Thus, there is a total of four assay tubes for each point (two tubes for each curve). Sample determinations are read from the average of the two curves.

2. Dry down all standards under air in 37˚C water bath.

3. Dilute samples with 10 megOHM H_2O such that 100 µl of sample will fall in the 0.02-2.0 ng range.

4. Incubate diluted samples in 37˚C water bath for 30 minutes. Add 100 µl warm (37˚C) assay buffer to all sample tubes. Add 200 µl assay buffer to standard curve tubes with the following exceptions: 1.0 ml assay buffer to total count tubes; 0.3 ml assay buffer to non-specific binding tubes. Vortex all tubes and incubate at 40˚C for 30 minutes.

5. Place rack of assay tubes into ice bath.

6. Mix antiserum with magnetic stirring rod. Pipette 100 µl antiserum into all standard and sample tubes except total counts and NSB (Non-specific binding). Vortex briefly. Wait 20 min. in an ice bath.

7. Pipette 100 µl assay tracer into all tubes. Vortex briefly. Cover with tin foil and incubate at 4˚C for at least 4 hours (preferably overnight).

Separation of Bound & Free Progesterone:

1. At end of incubation transfer rack of tubes into a fresh ice bath and add 0.75 ml cold dextran-coated charcoal. *Except to total counts tubes. Charcoal should be kept in an ice bath and under constant stirring while adding to tubes. *Add the charcoal as rapidly as possible and not to more than 30 tubes at one time to avoid stripping.

2. **Promptly** vortex each tube **briefly.** Incubate samples for 4 min., starting when the first tube receives the charcoal. -Centrifuge 10 min. at 3000 xg (3500 RPM) in **refrigerated centrifuge.**

3. Decant supernatant fraction of each tube into numbered scintillation vial inserts. Do not disturb charcoal pellet at the bottom.

4. Add 4 ml counting cocktail, vortex at high speed and count for at least 1 min. *Charcoal suspension picks up free progesterone leaving the bound progesterone on the antibody in solution to be counted. *If only PPO and Toluene are used in counting cocktail, samples must sit for at least 5 hr. to allow the cocktail to extract the ^{3}H-progesterone out of the sample.

Preparation of Solutions:

1. Assay Buffer. Do not store for more than 2 weeks

$NaH_2PO_4 \cdot H_2O$	5.38 gm
$Na_2HPO_4 \cdot 7H_2O$	16.35 gm
NaCL	9.0 gm
Na Azide	1.0 gm
Bovine Gamma Globulin	
(Fraction II) or Gelatin	1.0 gm

    Add 900 ml 10 megohm H_2O and mix on a lowly heated magnetic stirrer until all contents are completely dissolved. Maintain stirring and adjust pH to 7.0 with 1.0 N NaOH. Adjust volume to 1 liter with 10 megohm H_2O.

2. Charcoal Suspension. Do not store for more than 2 wks.

    20 mg Dextran T-70®
    200 mg prewashed Norit A neutral charcoal

    Add 100 ml assay buffer and magnetically stir for about 20 min. at high speed while placed in an ice bath.

3. Antiserum. Always dilute with assay buffer. The activity of the antibody determines amount of dilution. Always make up final dilution the day of the assay.

4. Assay Tracer. Pipette 20 µl tritiated progesterone (^{3}H-P_4) into counting vial. Dry down. Add 10 ml assay buffer. Vortex 30 seconds at high speed. Place in 37°C water bath 20 minutes. Refrigerate before using.
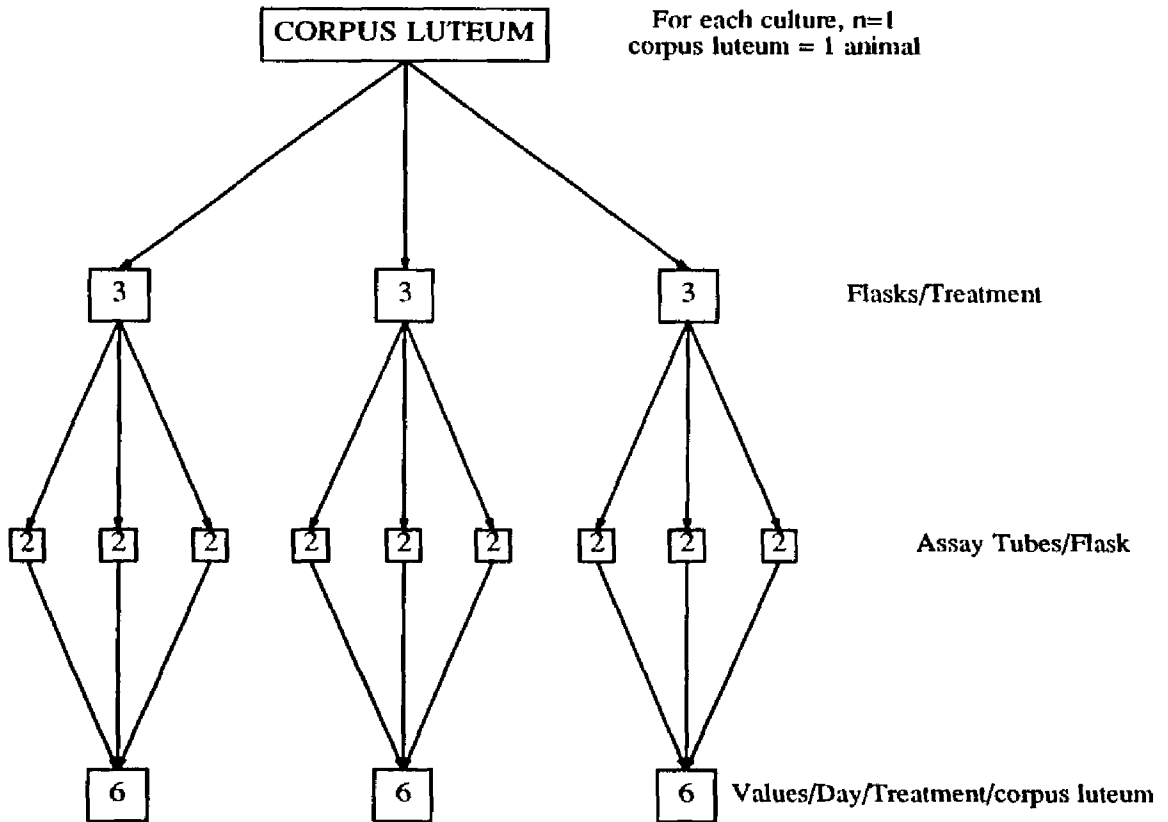
5. Recovery Tracer. Pipette 1.0 ml assay tracer into counting vial. Add 9.0 ml assay buffer. Vortex 30 seconds and refrigerate.

6. Counting Cocktail.

    1.32 gm PPO
    1 liter toluene

    Stir on magnetic stirrer for about 15 minutes.

APPENDIX C

CORPUS LUTEUM

For each culture, n=1
corpus luteum = 1 animal

3   3   3   Flasks/Treatment

2 2 2   2 2 2   2 2 2   Assay Tubes/Flask

6   6   6   Values/Day/Treatment/corpus luteum

Example for 1 treatment in an 8 day culture for 1 experiment:

Treatment		Flasks		Assay Tubes		Day		Animals		Total values
1	×	3	×	2	×	4	×	6	=	144

145