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The role of prostaglandins and the phosphatidylinositol signalling system in the regulation of the primate corpus luteum

Houmard, Brenda Sue, Ph.D.
The Ohio State University, 1992
THE ROLE OF PROSTAGLANDINS AND THE PHOSPHATIDYLINOSITOL SIGNALLING SYSTEM IN THE REGULATION OF THE PRIMATE CORPUS LUTEUM

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

The Ohio State University

1992

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Department of Dairy Science
To my loving husband, Todd
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Houmard BS, Ottobre JS, 1989. Progesterone and prostaglandin production by primate luteal cells collected at various stages of the luteal phase: modulation by calcium ionophore. Biol Reprod 41:401-8

Houmard BS, Harder JD, Ottobre JS, 1989. Prostaglandin F in reproductive tissues collected during the luteal phase of the estrous cycle and at parturition in Virginia opossums (Didelphis virginiana). Theriogenology 32:817-26


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FIELDS OF STUDY

Major Field: Dairy Science
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INTRODUCTION

The corpus luteum (CL) is a transient endocrine gland derived from the remnants of the ovulated follicle. The primary secretory product of the CL is progesterone. The uterus is the principle target organ of progesterone. Under the influence of progesterone, the uterine mucosa undergoes a transition from the estrogen-driven proliferative phase to the secretory phase (1). The endometrial secretions induced by progesterone are necessary to support the conceptus until placentation occurs. Progesterone also acts on the myometrium to inhibit contractions. The importance of luteal progesterone production for normal reproductive function is evident from the observations that luteectomy induces abortion in the fertile cycle (2, 3) and menstruation in the nonfertile cycle. These effects of luteectomy can be prevented by administration of exogenous progesterone (3). Since adequate function of the corpus luteum is essential to the establishment and maintenance of early pregnancy, advances in the understanding of primate luteal function provide a basis for improved fertility control in the woman.
The menstrual cycle can be divided into a follicular phase and a luteal phase. During the follicular phase, the ovarian follicle is the dominant structure on the ovary. The follicle consists of an avascular granulosal cell layer, which surrounds the oocyte, and a highly vascular thecal cell layer. These two components of the follicle are separated by a basement membrane. The functional interaction between the thecal cell layer and the granulosal cell layer (4, 5) results in the production of estrogen, the main secretory product of the follicle.

The luteinizing hormone (LH) surge which induces ovulation of the mature follicle also induces morphological and biochemical changes in the cells of the follicle. This process of luteinization is functionally characterized by a shift from predominant estrogen to predominant progesterone synthesis. In the rat, these functional changes result from alterations in several steroidogenic enzymes. The mRNA and protein for aromatase and 17α-hydroxylase decrease while those for the P450 side-chain cleavage (P450scc) enzyme increase during luteinization in the rat (6, 7). Similarly, P450scc mRNA levels are elevated during luteinization of the primate follicle (8). In contrast to many species, the primate CL retains the ability to synthesize estrogens. Therefore, it is not surprising that the mRNA levels for aromatase and 17α-hydroxylase do not decline in the primate CL (8).
The morphological changes which occur during luteinization of the primate follicle (9, 10) include breakdown of the basement membrane separating the granulosal and thecal cell layer and vascularization of the granulosal cell layer. Unlike many species, the primate CL remains somewhat compartmentalized (9-11). "True luteal cells" occupy the central portion of the CL. "Paraluteal cells" are found on the periphery of the CL and in invaginations into the more central region. During luteinization, the granulosal cells increase in size and accumulate increased amounts of lipid droplets. Thus, although the CL is derived from the preceding follicle, substantial biochemical and morphological changes occur during the transformation from follicle to CL.

In the primate (9, 12), as well as other species (13-18), luteal cells appear to be derived from both the thecal and granulosal cells of the follicle. Like the follicle, the CL consists of at least two types of steroidogenic cells in several species (14, 19, 20), including the primate (21-23). The distinction between luteal cell types, in terms of morphology and function, has been studied most thoroughly in the ewe (24) and cow (25). In these species, luteal cells are typically classified by size (small and large) or cell of origin (thecal-lutein and granulosal-lutein).

As described in the ewe (24) and cow (25), small luteal cells are spindle-shaped cells (20 μm or less in diameter)
with an irregular-shaped nucleus. In contrast, large luteal
cells are typically polyhedral cells (greater than 20-22 μm
in diameter) with a large, centrally located nucleus.
Functionally, small cells display low basal progesterone
production but are highly responsive to LH stimulation.
Large cells secrete high levels of progesterone under basal
conditions, are relatively unresponsive to gonadotropin
stimulation, and are the primary site for luteal relaxin
(26) and oxytocin (27) synthesis. In addition, the large
cells appear to be the primary target for the luteolysin in
these species, prostaglandin (PG) F₂α (28).

The origin of the luteal cell types in these species
also appears to be somewhat distinct. Early morphological
studies suggested that the small and large luteal cells were
derived, respectively, from the thecal and granulosal layers
of the follicle. Using monoclonal antibodies to granulosal
and thecal cell surface antigens, Alila and Hansel (29)
examined this hypothesis in the bovine CL. In the early
luteal phase, small cells bind thecal-specific antibodies;
whereas, large cells primarily bind granulosal-specific
antibodies. However, as the luteal phase progresses, large
cells show an enhanced ability to bind thecal-specific
antibodies. Thus, the hypothesis that small and large
luteal cells of the domestic species are derived from thecal
and granulosal cells, respectively, appears partially
correct. However, it is possible that some small cells become large cells during the luteal phase.

Recently, the distribution and classification of various cell types in the primate CL have been described. Based on size classification, the CL of the rhesus monkey has been reported to consist of three populations of luteal cells (21): small ($\leq 15 \mu m$), medium (16-20 \mu m) and large (>20 \mu m) cells. As observed in the domestic species, basal progesterone is substantially greater in large luteal cells as compared to small luteal cells (21, 30). In the early luteal phase, both cell types respond to gonadotropin with increased progesterone production (30). Whereas the large luteal cells retain gonadotropin-responsiveness throughout the luteal phase, this responsiveness in small cells is lost by mid-luteal phase. Unfortunately, the functional characterization of the medium cells from the macaque CL has been impeded by the inability to purify this population.

Functional studies of small (5-20 \mu m, a population of cells with a size distribution roughly equivalent to small plus medium macaque luteal cells) and large (>20 \mu m) cells from the human CL have been performed (22). Basal progesterone production is greater in the large, as compared to the smaller, human luteal cells (22). However, the smaller human luteal cells were more responsive to gonadotropin than the large cells (22). Thus, the functional differences between subtypes of primate luteal cells exist and are
similar in some respects to those found in the domestic species.

Because the luteal cell subpopulations have only recently been identified in the primate, the localization of peptide hormone production and responsiveness to potential luteolysins in the cell types of the primate CL have not been assessed. The origin of the subpopulations of primate luteal cells also remains to be determined. The histological arrangement of the "true luteal" and "paraluteal" cells has led to the speculation that the larger, "true luteal", cells are granulosal-derived and the smaller, "paraluteal", cells are thecal-derived (9, 12). However, further investigations are necessary to confirm this hypothesis.

As alluded to previously, hypophyseal gonadotropin (LH) is essential for the support and function of the primate CL. The essential role of LH in luteal support is evident from the observations that hypophysectomy (31) or administration of either LH antiserum (32) or GnRH antagonists (33, 34) leads to premature cessation of primate luteal function. These effects of hypophysectomy and GnRH antagonists can be reversed by exogenous gonadotropin (31, 33). Thus, LH plays a critical role in the formation and maintenance of the primate corpus luteum.

Although LH is considered to be the primary luteotropin in the primate CL, a luteotropic role for PGE_{2}, PGI_{2} and
PGD$_2$ has been postulated. This is based on observations which show stimulatory effects of these PGs on in vitro progesterone production by primate luteal cells (35-40). An obligatory role for PGs in the support of the primate CL is substantiated by the studies of Sargent and colleagues (41). This study demonstrated that intraluteal infusion of an inhibitor of PG synthesis beginning at mid-luteal phase induces premature luteolysis. Although further studies with blockers of specific PGs are necessary, the study of Sargent and others does support the hypothesis that some PGs may play a luteotrophic role in the primate CL.

The process of luteolysis in the primate is less understood than luteotropism. Luteolysis in the laboratory and domestic species appears to result from the release of uterine PGF$_{2\alpha}$, which acts on the corpus luteum to initiate functional and structural luteal regression (42, 43). The evidence supporting this hypothesis includes studies which have demonstrated inhibitory responses of the CL to exogenous PGF$_{2\alpha}$ (44-50). In addition, the luteal phase of the domestic and laboratory species can be prolonged by hysterectomy (51-57), immunization against PGF$_{2\alpha}$ (57-60) or inhibition of uterine PGF$_{2\alpha}$ synthesis (57, 61-63). Therefore, uterine PGF$_{2\alpha}$ is the accepted luteolysin in most domestic and laboratory species.

The exact mechanism by which PGF$_{2\alpha}$ induces luteal regression in these species remains unclear. Possible
mechanisms include decreases in luteal blood flow (64, 65),
direct or indirect cytotoxic effects (66, 67), decreases in
luteal LH receptors (68-69) and interference at various
sites in the steroidogenic pathway (70-74). Further studies
are necessary to fully understand the mechanism of action of
PGF$_{2a}$ on the CL.

A uterine factor does not appear to be responsible for
luteal regression in the primate. Hysterectomy in several
primate species does not alter luteal function and/or
lifespan (75-78). Considering the essential role of LH in
primate luteal function and the observation that the
frequency of LH pulses declines during the luteal phase of
primates (79, 80), it has been hypothesized that primate
luteal regression results from a loss of gonadotropin
support.

To address this hypothesis, Zeleznik and Hutchison
utilized monkeys whose endogenous control of gonadotropin
secretion was blocked by hypothalamic lesions. Exogenous
GnRH could then be administered to precisely control the
pattern of gonadotropin secretion and to support normal
luteal function. Using this model, the requirement of LH in
the maintenance of the primate CL was confirmed.
Termination of exogenous GnRH administration led to a
decline in serum progesterone levels and menses (81).
However, several lines of evidence from studies of this
nature suggest that normal luteal regression does not result
from declining gonadotropic support. Maintenance of a fixed, high-frequency, regimen of GnRH does not prevent luteal regression (81, 82). Thus, primate luteal regression can occur in the absence of a decline in LH pulse frequency. In addition, initiating a low-frequency GnRH regimen, like that observed in the late luteal phase, in the early luteal phase does not result in premature luteal regression (83). These results suggest that regression of the primate CL is not due to a decline in LH pulse frequency.

In 1973, Knobil (84) proposed that luteolysis in the primate resulted from a substance produced within the corpus luteum or other parts of the ovary. Such a substance would likely act directly on ovarian tissue since subtle variations in gonadotropic secretion do not alter luteal lifespan (81-83). Recent studies of primate luteal regression have focused on the hypothesis proposed by Knobil. Estrogen has been suggested to be a potential luteolysin in the primate. Both peripheral (85) and ovarian (86) estrogen levels increase near the time of luteal regression. In addition, exogenous estrogens induce premature luteal regression in the primate (87, 88). However, the role of estrogen in spontaneous luteal regression has not been substantiated. As mentioned, in the model of Hutchison and Zeleznik LH secretion can be controlled with exogenous GnRH. In monkeys so treated, estrogen does not cause premature luteolysis, even when the
LH pulse frequency is low (89). In addition, suppression of luteal estrogen levels with an aromatase inhibitor does not change the lifespan of the corpus luteum (90). Furthermore, Hild-Petito and colleagues (91) were unable to detect estrogen receptor in the primate CL. These data suggest that estrogen is not a physiological mediator of primate luteal regression. Estrogen-induced luteolysis may result from a pharmacological inhibition of pituitary LH secretion.

Other proposed mediators of primate luteal regression have included oxytocin and GnRH. Oxytocin is a peptide hormone that appears to play a role in luteal regression in domestic species, such as the sheep. Immunization of ewes against oxytocin delays luteal regression (92, 93). The primate CL produces oxytocin (94, 95) and possesses oxytocin binding sites (96). Although the effects of oxytocin on primate luteal cells in vitro are controversial (97, 98), intra-luteal infusion of oxytocin causes luteal regression in the rhesus monkey (99). Additional studies, including immunization experiments like those performed in the sheep, are required to determine whether oxytocin mediates luteal regression in the primate.

GnRH is a hypothalamic hormone which, in addition to its effects on the pituitary, has been shown to directly inhibit progesterone production by the rat CL (100, 101). The rat CL possesses high-affinity GnRH receptors (100), and an endogenous GnRH-like protein has been isolated from the
rat ovary (102). A similar protein has been isolated from the human ovary (103). Although of much lower affinity than those of the rat CL, the primate CL has been shown to possess GnRH receptors (104). Given the potent luteolytic effects of in vivo GnRH in women (105), it is important to investigate the direct effects of GnRH on the primate CL. Other studies, however, have been unable to demonstrate direct effects of GnRH on human luteal progesterone production (106-109). Therefore, the hypothesis that GnRH acts at the ovarian level to induce luteolytic responses in the primate is not convincing.

Recently, the hypothesis that luteolysis in the primate may result from locally-produced, rather than uterine, PGF$_{2\alpha}$ has received a great deal of attention. One objective of this dissertation is to explore the notion that luteal prostaglandins, including PGF$_{2\alpha}$, are involved in the control of the primate CL.

In addition to characterizing the role of various extracellular agents in luteal regulation, it is important to understand the intracellular mechanisms by which these substances alter luteal function. Several general signalling mechanisms have been described in a variety of tissues and cell types. These include pathways linked to adenylate or guanylate cyclase activity, phosphatidylinositol hydrolysis, or tyrosine kinase activity. Hormones can also alter ion channel status and
membrane potential. These signalling mechanisms are typically initiated by binding of the extracellular signal to a plasma membrane receptor. Other agents, such as the steroid hormones, bind to cytosolic and/or nuclear receptors to control DNA transcription in the target cells. It is important to emphasize that, although described as separate pathways, these systems interact with one another.

Of these signal transduction pathways, the adenylate cyclase system has been most thoroughly studied in the CL. The receptor for LH on the luteal cell surface is coupled to adenylate cyclase within the luteal cell (110). Activation of adenylate cyclase increases intracellular concentrations of cAMP. Cyclic AMP activates a cAMP-dependent protein kinase (protein kinase A) (111). Enhanced activity of protein kinase A appears to increase luteal progesterone production via phosphorylation of enzymes or other proteins essential for steroid production (see 112 for review). The enzymes regulated by protein kinase A include those required to increase cytosolic cholesterol levels (cholesterol esterase) and to convert cholesterol to pregnenolone (P450scc enzyme). The luteotrophic effects of PGE$_2$ and PGI$_2$ also appear to be coupled to activity of adenylate cyclase in the primate CL (37, 38, 113, 114). Thus, the adenylate cyclase pathway is the major transducer of luteotrophic signals.
The existence and role of other signalling systems in the CL, particularly those mediating luteal regression, have only recently been investigated. Since the phosphatidylinositol second messenger system has been proposed to be a mediator of luteal regression in other species (115, 116), a second objective of this dissertation is to examine this system in the primate CL. Activation of the phosphatidylinositol system results in the production of inositol triphosphate and diacylglycerol, which subsequently increase intracellular free calcium concentrations ([Ca$^{2+}$]$_i$) and activate protein kinase C. As described in subsequent chapters, studies were designed to assess the effects of agents which increase [Ca$^{2+}$]$_i$ and activate protein kinase C on primate luteal function. In addition, various physiological agents were tested for an ability to activate the phosphatidylinositol pathway in the primate CL.

Since extension of luteal function is critical for maintenance of early pregnancy, it is important to understand the mechanism by which the conceptus signals its presence and delays luteolysis. In primates, maternal recognition of pregnancy results from the production of chorionic gonadotropin (CG). CG is a glycoprotein which is structurally similar to LH. LH and CG bind to the same receptor on primate luteal cells (117). Thus, it is not surprising that CG is functionally similar to LH (118). Exogenous CG is used in experimental protocols to mimic the
events in early pregnancy that lead to luteal rescue. In addition, CG is used in in vitro experiments to investigate gonadotropin-stimulated progesterone production.

CG secreted from the embryonic trophoblast is first detected in the peripheral serum near the time of implantation (119, 120). The hypothesis that CG is the principal signal responsible for maternal recognition of pregnancy in primates is strongly supported. First, the rise in CG and progesterone following conception are temporally associated (121). Second, administration of hCG in the mid-luteal phase of the menstrual cycle extends luteal lifespan and elicits a pattern of progesterone production similar to that of actual early pregnancy (122, 123). Finally, immunization against CG prevents pregnancy in primates, without altering normal cyclicity (124-126). In some studies, this induced infertility can be reversed with exogenous progesterone (127). Although it has been known for some time that CG mediates maternal recognition of pregnancy in primates, the mechanism by which CG rescues the CL from regression remains unclear. Perhaps, CG prevents the appearance of the luteolytic signal. Alternatively, CG may simply override the luteolytic mechanism with its luteotropc actions. Some of the studies described in this dissertation were designed to address this question.

In an effort to increase the understanding of primate luteal regulation, our laboratory has focused attention on
the role of prostaglandins and the phosphatidylinositol signalling pathway in the control of the primate CL. Advances in this area may provide a basis for improved fertility control in the woman.

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The mechanisms involved in the regulation of the primate CL, particularly those responsible for luteal regression, are poorly understood. Uterine prostaglandins influence CL function in several nonprimate species. Although the uterus is not necessary for normal luteal function and lifespan in the primate, prostaglandins from other sources may influence the CL. To assess the role of luteal prostaglandins in the regulation of the primate CL, studies were designed to characterize changes in luteal prostaglandin production during the menstrual cycle.

In addition to identifying the extracellular signals involved in primate luteal regulation, it is important to understand which signal transduction pathways mediate the effects of these extracellular signals. The role of the adenylate cyclase pathway in mediating the luteotropic effects of gonadotropin is well-documented. However, the role of other second messenger systems in luteal regulation has not been extensively studied, particularly in the primate. PGF$_{2\alpha}$, a possible luteolysin in the primate, activates the phosphatidylinositol signalling pathway in the CL of several nonprimate species. Therefore, several
studies were designed to investigate the role of this signalling pathway in the control of primate luteal function. The effects of agents which mimic events induced by activation of the phosphatidylinositol pathway on primate luteal function were examined. In addition, studies were designed to characterize and compare the ability of luteolytic and luteotropic agents to activate this signalling pathway. In an effort to better understand the rescue of the CL by chorionic gonadotropin in early pregnancy, the ability of in vivo hCG to alter potential luteolytic mechanisms was examined.
CHAPTER I
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Prodgerone and Prostaglandin Production by
Primate Luteal Cells Collected at Various Stages of the Luteal
Phase: Modulation by Calcium Ionophore

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ABSTRACT

Prostaglandins (PG) are produced by the corpus luteum (CL) of the rhesus monkey and may be involved in
luteal regulation. Intracellular calcium has also been implicated as a mediator of luteolysis in domestic and
laboratory species; however, its role in primate luteal function has not been investigated. The objectives of this
study were to characterize temporal changes in basal and stimulated luteal PC production by CL of rhesus
monkeys, and to examine the effects of calcium ionophore (Cal) on basal and gonadotropin-stimulated progesterone (P) production by the CL. CL were collected at various times after the estimated day of the luteinizing hormone (LH) surge: 5 days (early luteal phase, n = 4), 8-10 days (mid-luteal phase, n = 8), and 12-14 days (late luteal phase, n = 5). Dispersed luteal cells were incubated in the absence and presence of Cal, or with human chorionic gonadotropin (hCG) plus Cal at 37°C for 8 h. PG and P concentrations in the medium were measured by radioimmunoassay. PGE$_2$ and 6-keto-PGF$_{1\alpha}$ production decreased (p<0.05) from early luteal phase to mid-luteal phase and remained lower (p<0.05) during late luteal phase for all treatment groups. PGF$_{2\alpha}$ production decreased (p<0.05) from early to mid-luteal phase and rebounded in late luteal phase to the same level (p>0.05) found in early luteal phase. Cal stimulated (p<0.05) basal PC production. The degree of stimulation was similar throughout the luteal phase (p>0.05). Cal inhibited basal P production in a dose-dependent manner (p<0.01). In addition, Cal decreased the maximum response, in terms of P production, to hCG (p<0.05). This study provides evidence consistent with a role for luteal PGs and intracellular calcium in primate luteal regulation. Luteotropic PGs, PGE$_2$ and PGI$_2$ (measured by 6-keto-PGF$_{1\alpha}$ production), were found in the highest concentrations in early luteal phase, when, from other studies, they appear to exert the most potent luteotropic effect. PGF$_{2\alpha}$, a luteolytic PG, was found at high levels in both early luteal phase and late luteal phase; however, from other work, its expected luteolytic effect is absent during early luteal phase. Inhibition of basal and hCG-stimulated luteal P production by Cal implies a potential role for intracellular calcium in primate luteal regulation.

INTRODUCTION

Uterine prostaglandins (PG) influence corpus luteum (CL) function in domestic and laboratory animals (Horton and Poyser, 1976; Inskeep and Murdoch, 1980). PGF$_{2\alpha}$ is often considered to be luteolytic (Horton and Poyser, 1976), and other PGs, such as PGE$_2$ (Marsh and LeMaire, 1974; Huecksteadt and Weems, 1978) and PGI$_2$ (Milvae, 1986), are considered to be luteotropic. Although in primates the uterus is not necessary for normal luteal function and lifespan (Neill et al., 1969; Castracane et al., 1979), PGs from other sources may influence the CL. Exogenous PGs affect luteal production of progesterone (P) in primates in vitro (Stouffer et al., 1979; Dennefors et al., 1982; Patwardhan and Lanier, 1984) and in vivo (Aulenta et al., 1984). CL of rhesus monkeys produce PGs in vitro (Johnson et al., 1988), and inhibition of local PG synthesis in vivo alters luteal P production and luteal lifespan (Sargent et al., 1988). Luteal PGs may play a role in regulation of primate CL.

Changes in luteal PG production have been observed during the estrous cycle in cattle (Milvae and Hansel, 1983), and the content of PGs in human CL changes throughout the luteal phase (Challis et al., 1976; Shutt et al., 1976; Vijayakumar and Walters, 1983; Patwardhan and Lanier, 1985). These changes may be important in regulating luteal function. Balmaceda et al. (1979) reported changes in P production in rhesus...
monkeys during the luteal phase by measuring PGs in medium after 10 min of exposure to luteal tissue. However, the inferred luteal production of PG may have been due to leaching of PGs from whole CL into incubation medium. PG production by human CL at various stages of the luteal phase has also been reported (Challis et al., 1976; Patwardhan and Landthier, 1985), but interpretation of these studies is difficult due to a wide range in CL age within each group and an imprecise estimate of CL age. Therefore, changes in PG production during the primate luteal phase should be more rigorously examined.

Increases in intracellular calcium have been implicated as a mediator of luteolytic hormones in rodents and domestic animals (Behrman et al., 1986). Treatment with calcium ionophore (Cal), which increases intracellular calcium, inhibits P secretion (Behrman et al., 1979) and causes a decrease in luteinizing hormone (LH)-stimulated cyclic adenosine monophosphate (cAMP) accumulation (Dorflinger et al., 1984) in rat luteal cells. Increased calcium concentrations also inhibit the responsiveness of adenylate cyclase to gonadotropin in isolated luteal membranes of primates (Rojas et al., 1989). Cal stimulates PG production by luteal cells of rhesus monkeys (Johnson et al., 1988); however, a definitive role for calcium in regulation of primate luteal function has yet to be elucidated.

The objectives of the current study were (1) to characterize temporal changes in basal and Cal-stimulated PG production by CL of nonpregnant rhesus monkeys and (2) to examine the effects of Cal on basal and gonadotropin-stimulated P production by the CL.

MATERIALS AND METHODS

Treatment of Animals

The housing and general care of rhesus monkeys at The Ohio State University was described previously (Johnson et al., 1988). Monkeys were checked daily for menses, and menstrual records were maintained (onset of menses = Day 1 of the menstrual cycle). Animals exhibiting regular cycles of approximately 28 days were used in the current study. Blood samples were collected daily by femoral venipuncture at 0800-1000 h from Day 8 of the menstrual cycle until luteectomy. The serum was stored at -20°C until assayed for hormones. On Day 14 of the cycle, serum concentrations of 17β-estradiol were determined by a rapid radioimmunoassay (RIA) (Ottobre et al., 1984). The day of the LH surge was estimated to occur 1 day before the precipitous decline in estrogen concentration that followed the midcycle estrogen peak (Channing, 1980). Following these criteria permits accurate and precise estimation of CL age.

CL were collected via midventral laparotomy at various times after the estimated day of the LH surge: 5 days (early luteal phase, n = 4), 8-10 days (mid-luteal phase, n = 8), and 12-14 days (late luteal phase, n = 5). Monkeys were anesthetized (Johnson et al., 1988) before surgery with ketamine hydrochloride (Bristol Laboratories, Syracuse, NY; 10-15 mg/kg, i.m.) supplemented with atropine sulfate (ESI Pharmaceuticals, Cherry Hill, NJ; 0.02 mg/kg, i.m.) and sodium pentobarbital (Butler, Columbus, OH; 8-12 mg/kg, i.v.). The CL were excised from the ovaries by blunt dissection and immediately placed in Ham's F-10 medium (Sigma, St. Louis, MO) at 4°C for transport to the laboratory.

Preparation and Treatment of Tissue

Dispersed luteal cells were prepared by a combination of gentle mechanical agitation and digestion with 0.25% collagenase (Worthington Biochemical, Freehold, NJ) and 0.02% deoxyribonuclease (Sigma) (Johnson et al., 1988). Cell viability, as determined by trypan blue exclusion, was >95%. Suspensions of dispersed luteal cells were incubated in a gyratory shaker bath at concentrations of 50,000 cells per 250 µl Ham's F-10 medium at 37°C in an atmosphere of 95% O2:5% CO2. Incubation times of 0 h (no incubation) and 8 h were used. Initial content of P and PG was the concentration at 0 h. Cells collected from early, mid-, and late luteal phases, were incubated without treatment and with calcium ionophore (A23187, Sigma) at a concentration of 1 µM. This dose of Cal was chosen since it stimulated PG production maximally (Johnson et al., 1988). To characterize the dose-response to Cal, in terms of P production, cells collected during mid-luteal phase were incubated with Cal at various concentrations (0.05, 0.1, 0.5, 1, 5, and 10 µM). In addition, we examined the effect of Cal on gonadotropin-stimulated P production. Cells from mid-luteal phase were incubated with purified human chorionic gonadotropin (hCG; CR 125; 11,900 IU/mg, NIADDK) at concentrations of 0.05, 0.5, 5, 50, and 500 ng/ml in the absence and presence of 5 µM Cal. A dose of 5 µM Cal was chosen for these tests since that dose decreased P production maximally (cur-
rent study). Cells and medium were frozen at -20°C after incubation. Cells and medium were separated by centrifugation at 160 × g prior to RIA.

**RIAs**

RIAs were used to measure concentrations of P, PGE₂, PGF₂α, and 6-keto-PGF₁α (the stable metabolite of PGF₁₂) in samples of F-10 medium incubated with dispersed cells (Johnson et al., 1988). Specific and proven antibodies for P (Dr. Gordon Niswender, Colorado State University), PGE₂ (Dr. Harold Behrman, Yale University), PGF₂α and 6-keto-PGF₁α (Dr. Richard Fertel, The Ohio State University) were donated. Cross-reactivities of PG antibodies with similar compounds have been reported to be <0.1%, except for the PGF₂α antibody, which cross-reacts 15% with PGF₁α (Fertel et al., 1981). Advanced Magnetics (Cambridge, MA) donated the PG standards. Radioactive steroids were purchased from New England Nuclear (Boston, MA), and radioactive PGs were purchased from Amersham (Arlington Heights, IL).

The validation of these assays has been reported previously (Johnson et al., 1988). Exogenous treatments did not interfere with the RIAs. Coefficients of variation within and between assays were as follows: P, 11.06% and 14.22%; PGF₂α, 6.44% and 5.12%; PGE₂, 4.26% and 10.55%; and 6-keto-PGF₁α, 8.82% and 5.94%.

**Statistical Analyses**

Analyses of variance were used to make the following comparisons. P and PG concentrations in medium following 0 h incubation (no treatment) and 8 h incubation (no treatment and 1 μM Cal treatment) were compared between age groups. The PGE₂/PGF₂α and 6-keto-PGF₁α/PGF₂α ratios for each treatment and the number of cells recovered from luteal dispersion were also compared between age groups. The difference between product concentrations following 8 h incubation with no treatment and those following treatment with Cal was computed to determine the magnitude that Cal stimulated PG production or inhibited P production. These differences were then compared between age groups. The fold change by Cal (concentration at 8 h, no treatment/concentration at 8 h, 1 μM Cal) was also compared between age groups. The maximum response to hCG, in terms of P production, was compared for cells treated with hCG in the absence and presence of Cal. To reduce heterogeneity of variance between groups, data were sometimes transformed to natural logarithms.

For all of the following comparisons we used analyses of variance for a randomized complete block design with individual CL as the block. P and PG concentrations in medium following 8 h incubation with no treatment were compared to concentrations following treatment with 1 μM Cal. P concentrations in medium were compared between doses of Cal. The pattern of P production in response to a range of hCG doses was compared to that following treatment with 5 μM Cal plus the hCG doses.

Correlation coefficients between basal production of P and basal production of each of the PGs were calculated.

**RESULTS**

Figures 1–4 illustrate mean PG and P concentrations in medium incubated with CL collected at various stages of the luteal phase. For each stage, concentrations of PG or P at 0 h (no treatment) and 8 h (in the absence and presence of Cal) are represented.

PGE₂ concentrations (Fig. 1) decreased (p<0.05) from early luteal phases to mid-luteal phase and remained suppressed (p<0.05) during late luteal phase for all treatment groups. After 8 h incubation without treatment, concentrations of PGE₂ in late luteal phase were lower (p<0.05) than in early luteal phase, but were higher (p<0.05) than in mid-luteal phase. For all treat-
Incubation time (h): 0, 6, 12, 24
FIG. 2. Concentrations of 6-keto-prostaglandin (PGF1\(_{12a}\)) (mean ± SE) in medium incubated with corpora lutea collected during early, mid-, and late luteal phase. See Figure 1 for explanation of experimental conditions. Means within treatment group with different letter designations were significantly different (p<0.05). Cal, calcium ionophore.

Incubation time (h): 0, 6, 12, 24
FIG. 3. Prostaglandin (PGF2\(_{2a}\)) concentrations (mean ± SE) in medium incubated with corpora lutea collected during early, mid-, and late luteal phase. See Figure 1 for explanation of experimental conditions. Means within treatment group with different letter designations were significantly different (p<0.05). Treatment with calcium ionophore (Cal) significantly decreased (p<0.01) PGF2\(_{2a}\) concentrations after 8 h incubation throughout the luteal phase.

m ent groups, 6-keto-PGF1\(_{12a}\) concentrations (Fig. 2) decreased (p<0.05) from early luteal phase to mid-luteal phase and remained low (p<0.05) during late luteal phase. Concentrations of PGF2\(_{2a}\) in medium (Fig. 3) from untreated cells prior to incubation declined significantly (p<0.05) from early luteal phase to mid-luteal phase and late luteal phase. After 8 h incubation in the absence or presence of Cal, PGF2\(_{2a}\) concentrations decreased (p<0.05) from early luteal phase to mid-luteal phase and rebounded in late luteal phase to the same level (p>0.05) found in early luteal phase.

The PGE2/PGF2\(_{2a}\) ratios (Table 1) did not significantly differ across stage of the luteal phase for any treatment. The 6-keto-PGF1\(_{12a}\)/PGF2\(_{2a}\) ratios (Table 1) tended to be higher in early luteal phase than in mid-luteal phase and late luteal phase after incubation for 8 h without treatment (p = 0.06) and 8 h with 1 \(\mu\)M Cal (p = 0.07).

P concentrations (Fig. 4) did not differ across stage of the luteal phase at 0 h without treatment. However, P concentrations after 8 h, in the absence and presence of Cal, decreased (p<0.05) from early luteal phase to mid-luteal phase and late luteal phase. P concentrations were significantly correlated with concentrations of PGE2 (r = 0.737, p<0.01, n = 17) and 6-keto-PGF1\(_{12a}\) (r = 0.693, p<0.01, n = 17) across the luteal phase. No correlation was found between P and PGF2\(_{2a}\) concentrations (r = 0.337, p>0.05, n = 17).

Treatment with Cal significantly increased production of PGF2\(_{2a}\) (p<0.01) and PGE2 (p<0.01) and significantly decreased (p<0.01) production of P. For these comparisons, data were analyzed with individual CL as a block; therefore, variation between individual CL was removed. No significant change (p>0.05) was observed throughout the luteal phase in the magnitude of stimulation of PG production produced by Cal. In terms of P production, there was no alteration in fold change induced by Cal throughout the luteal phase, but Cal decreased P concentrations to a greater magnitude in early luteal phase than in either mid-luteal phase or late luteal phase (p<0.05).

Incubation time (h): 0, 6, 12, 24
FIG. 4. Progesterone concentrations (mean ± SE) in medium incubated with corpora lutea collected during early, mid-, and late luteal phase. See Figure 1 for explanation of experimental conditions. Means within treatment group with different letter designations were significantly different (p<0.05). Treatment with calcium ionophore (Cal) significantly decreased (p<0.01) progesterone concentrations after 8 h incubation throughout the luteal phase.
TABLE I. Prostaglandin (PG) ratios during the luteal phase.*

<table>
<thead>
<tr>
<th>PG</th>
<th>Stage of luteal phase</th>
<th>Early</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:PGF2α/PGF1α ¹</td>
<td>0 h, no tm</td>
<td>14.33 ± 3.31</td>
<td>10.63 ± 1.38</td>
<td>11.86 ± 2.39</td>
</tr>
<tr>
<td>8 h, no Cal</td>
<td>6.97 ± 1.36</td>
<td>8.92 ± 1.21</td>
<td>7.03 ± 2.22</td>
<td></td>
</tr>
<tr>
<td>8 h, 1 µM Cal</td>
<td>6.97 ± 1.58</td>
<td>9.81 ± 1.42</td>
<td>7.18 ± 1.75</td>
<td></td>
</tr>
<tr>
<td>6-keto-PGF1α/PGF2α</td>
<td>0 h, no tm</td>
<td>1.64 ± 0.29</td>
<td>0.94 ± 0.25</td>
<td>1.38 ± 0.17</td>
</tr>
<tr>
<td>8 h, no tm</td>
<td>1.12 ± 0.12</td>
<td>0.62 ± 0.13</td>
<td>0.62 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>8 h, 1 µM Cal</td>
<td>0.81 ± 0.04</td>
<td>0.54 ± 0.09</td>
<td>0.48 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

*Values expressed as mean ± SE.

¹PGE2/PGF2α ratios did not differ (p>0.05) across stage of the luteal phase for any treatment (tm).

²6-keto-PGF1α/PGF2α ratios tended to be higher in early luteal phase than in mid-luteal and late luteal phases after incubation for 8 h without treatment (p = 0.06) and 8 h with 1 µM Cal (p = 0.07).

The effects of Cal on P production by mid-luteal phase CL were examined more closely. Cal decreased concentrations of P in medium in a dose-dependent manner (Fig. 5; p<0.01). This decrease in P cannot be attributed to decreased cell viability, because viability was similar (p>0.05, n = 3) between untreated cells and those treated with 10 µM Cal. The maximum response to hCG, in terms of P production, was lower (p<0.05) after Cal treatment (Fig. 6). The pattern of hCG-stimulated P production tended to differ with the addition of Cal (Cal x dose of hCG, p = 0.07).

The number of cells recovered from luteal dispersion increased (p<0.01) from early luteal phase (1.08 ± 0.23 million, x ± SE) to mid-luteal phase (3.14 ± 0.29 million) and then declined (p<0.01) in late luteal phase (2.01 ± 0.43 million).

**DISCUSSION**

The results of this study demonstrate that production of PGs by CL from rhesus monkeys changed at various stages of the luteal phase. The temporal patterns of luteal production of PGs observed agree with those of similar studies in cattle (Milvae and Hansel, 1983; Rodgers et al., 1988). However, this issue is confusing in primates because different studies have produced some conflicting data. Discrepancies may be related to wide ranges in CL age in some studies, imprecise...
estimates of CL age, or inferred production of PGs by luteal tissue that could have been due to leaching of PGs into the incubation medium. Since the incubation system used in the current study was thoroughly validated to measure actual luteal production of PGs (Johnson et al., 1988), and since accurate and precise estimates of CL age were made, this study helps to clarify the issue of luteal PG production during the luteal phase of the primate menstrual cycle.

Concentrations of PGE_2 and 6-keto-PGF_1α, compounds that represent putative luteotropins, were highest in early luteal phase, decreased in mid-luteal phase, and remained depressed in late luteal phase in a manner similar to that observed for production of PGE_2 (Rodgers et al., 1988) and 6-keto-PGF_1α (Milvae and Hansel, 1983; Rodgers et al., 1988) in cattle. The notion of a luteotropic role for PGE_2 and PGF_2α in primate CL is supported by the significant positive correlations between luteal production of P and that of PGE_2 and 6-keto-PGF_1α (the stable metabolite of PGF_2α). Concentrations of PGE_2, a putative luteolysin, decreased from early luteal phase to mid-luteal phase but, in contrast to the luteotropic PGs, rebounded in late luteal phase to levels observed in early luteal phase. Although it appears that there is differential production of PGE_2 and PGF_2α during the luteal phase, no differences were observed in the PGE_2/PGF_2α ratio. The tendency for a decrease in the 6-keto-PGF_1α/PGF_2α ratio from early luteal phase to mid-luteal phase and late luteal phase agrees with the results from studies in cattle (Milvae and Hansel, 1983). Elevated levels of a luteolytic PG and depressed levels of luteotropic PGs during late luteal phase are consistent with a role for PGs in luteolysis.

Results of other studies suggest that the effects of PGs on primate luteal tissue also change as the luteal phase progresses. Correlating the changes in luteal response to PGs with changes in luteal PG production during the luteal phase may strengthen the notion of a role for PGs in luteolysis. PGE_2 was found in the highest concentrations in early luteal phase, the stage during which PGE_2 stimulates basal P production (Dennefors et al., 1982; Casper and Cottrell, 1984) and potentiates (Dennefors et al., 1982) gonadotropin-stimulated P and/or cAMP production. PGE_2 becomes less luteotropic (Hamberger et al., 1987) as the luteal phase progresses and luteal PGE_2 concentrations decrease. By late luteal phase, PGE_2 has no effect on basal P production (Stouffer et al., 1979; Hahlin et al., 1988) and inhibits gonadotropin-stimulated P production (Stouffer et al., 1979). Perhaps the modest increase in PGE_2 concentrations from mid-luteal phase to late luteal phase reflects a luteolytic role for PGE_2 at that time.

PGF_2α, a luteotropin in cattle (Milvae and Hansel, 1980), rabbits (Abramowitz and Birnbaumer, 1979), and goats (Band et al., 1986), has not been extensively studied in primates. Hamberger et al. (1980) have reported increases in basal cAMP production by human luteal tissue following treatment with PGF_2α in early luteal phase, the stage in which it was found in highest concentrations in this study (as reflected by concentrations of the major metabolite of PGF_2α, 6-keto-PGF_1α). PGF_2α also stimulates adenylyl cyclase in monkeys during mid-luteal phase (Molskness et al., 1987).

Although PGF_2α, a putative luteolysin, was found in high concentrations in early luteal phase (current study; Shutt et al., 1976), it has been shown to have no effect on basal or gonadotropin-stimulated and cAMP production at this stage (Hamberger et al., 1980; Dennefors et al., 1982; Bennegard et al., 1984). In fact, Stouffer et al. (1979) found PGF_2α to stimulate basal P production on Days 16–18 of the menstrual cycle (early luteal phase to mid-luteal phase). In mid-luteal and late luteal phases, PGF_2α becomes more luteolytic, as evidenced by its ability to inhibit gonadotropin-stimulated P and cAMP production (Hamberger et al., 1979, 1980; Stouffer et al., 1979; Dennefors et al., 1982; Bennegard et al., 1984). The absence of significant correlation across the luteal phase between in vitro P and PGF_2α concentrations is consistent with the disparity in the effects of PGF_2α on the CL at various stages of the luteal phase. Although the effects of PGF_2α are similar in mid-luteal and late luteal phases, depressed levels of PGF_2α in mid-luteal phase and rebounding PGF_2α levels in late luteal phase may ensure that luteolysis occurs at the appropriate time. Thus, a combination of changes in production of luteotropic and luteolytic PGs and changes in the responsiveness of luteal tissue to these PGs may play a role in controlling luteal function in primates.

An issue that seldom has been addressed in studies of luteal PG production is the possibility that cell types other than luteal cells are contributing to the observed PG synthesis. These cells may be endothelial cells, fibroblasts, macrophages, and/or pericytes (O'Shea et al., 1979). Studies have shown that these cell types are capable of synthesizing PGs (Myatt et al., 1975; Kurland and Bockman, 1978; Baenziger et al., 1979; Ali et al., 1980; Alhenc-Gelas et al., 1982). Therefore, the current and previous studies indicate that primate CL can produce PGs; however, further studies are neces-
sary to determine which cell types within the CL are responsible for this synthesis.

It has been suggested that calcium mediates the luteolytic effects of PGF$_{2\alpha}$ (Behrman et al., 1986). The antigo- gonadotropin effect (Dorflinger et al., 1984; Rojas et al., 1989) and decrease in luteal $P$ synthesis (Behrman et al., 1979) following Cal treatment support this hypothesis. This effect is thought to be mediated by release of intracellular calcium since blocking extracellular calcium influx has no effect on the action of PGF$_{2\alpha}$ (Lahav et al., 1983; Gore and Behrman, 1984).

The current study represents the first examination of the effects of calcium on $P$ production by primate luteal cells. The dose-dependent decrease of basal $P$ production and the reduced maximal response to hCG, in terms of $P$ production, are consistent with a role for calcium in luteolysis of primates. Similarly, calcium inhibited the responsiveness of adenylate cyclase to hCG in isolated luteal membrane of primates (Rojas et al., 1989). Although in the current study, fold change by Cal did not differ throughout the luteal phase, Cal decreased $P$ production to a greater magnitude in early luteal phase than in either mid-luteal phase or late luteal phase. Perhaps $P$ production in the later stages of the luteal phase is already suppressed by endogenous intracellular calcium release. This notion is supported by results from this study and others (Stouffer et al., 1977) showing decreased levels of basal $P$ production in mid-luteal phase and late luteal phase as compared to early luteal phase. However, further studies are required to elucidate the significance of decreasing responsiveness to Cal as the primate luteal phase progresses.

It is also interesting to note that intracellular calcium activates phospholipase $A_2$ (Hochachka, 1986), the enzyme that catalyzes the release of the PG precursor, arachidonic acid. This activation of phospholipase $A_2$ is probably responsible for the stimulation of PG production by Cal. It is possible that elevations in PG synthesis mediate the effects of Cal on $P$ production. However, since blocking PG production has no effect on luteal $P$ production during short-term incubation (Johnson et al., 1988), production of $P$ appears independent of PG synthesis in this experimental system. Thus, the acute effects of Cal on $P$ production may not be mediated by PGs. These observations do not rule out a chronic effect of local PGs on luteal $P$ production, particularly in light of data from cultured bovine luteal cells which demonstrate such an effect (Pate and Condon, 1984).

The pattern of in vitro production of $P$ in the current study does not reflect the typical peripheral pattern of $P$ observed in the menstrual cycle. Similarly, a poor correlation of these patterns is observed in other species in various reproductive states (Shala and Greenwald, 1982; Milvae and Hansel, 1983). However, the numbers of cells recovered during luteal dispersion increased ($p<0.01$) from early luteal phase to mid-luteal phase and then declined ($p<0.01$) in late luteal phase in a pattern resembling that of peripheral $P$. Similarly, in ewes, numbers of luteal cells increased during luteal development and decreased during luteal regression (Farin et al., 1986). Thus, the peripheral $P$ pattern of the menstrual cycle may be the result of changes in both the production of $P$ per cell and the number of cells per CL.

In summary, this study demonstrates that production of luteotropic and luteolytic PGs by primate CL changes throughout the luteal phase. Luteotropic PGs are found in the highest concentrations in early luteal phase, when they exert the most potent luteotropic effects and when $P$ production per cell is the highest. PGF$_{2\alpha}$, a luteolytic PG, is found at high levels in both early luteal phase and late luteal phase; however, its expected luteolytic effects appear only during mid- and late luteal phases. Therefore, coordination of luteal $P$ production and luteal responsiveness to PGs may be important in primate luteal regulation. The current experiments also demonstrated that treatments known to increase intracellular calcium inhibited basal and hCG-stimulated $P$ production by primate luteal cells and stimulated luteal PG production. Thus, changes in intracellular calcium may also be involved in the regulation of primate luteal function. The current data provide a framework for further investigation of the role of luteal PGs and intracellular calcium in primate luteal regulation.

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CHAPTER II

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The Effects of Elevation and Depletion of Intracellular Free Calcium on Progesterone and Prostaglandin Production by the Primate Corpus Luteum

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ABSTRACT

The role of the phosphatidylinositol second messenger system in luteal regulation has not been extensively studied, particularly in the primate. The objectives of this study were (1) to further characterize the response of the primate CL to the calcium ionophore A23187, in terms of intracellular free calcium concentrations ([Ca2+]i) and progesterone (P) production, and (2) to assess the effects of depletion, as well as elevating, available calcium on luteal P and prostaglandin (PG) production. The response to A23187 in terms of [Ca2+]i was measured by fura-2 fluorescence microscopy of single small and large luteal cells. A23187 significantly increased [Ca2+]i in both cell types (p < 0.01) P production (basal and hCG-stimulated) by dispersed primate luteal cells exposed to A23187 for various times (1-8 h), both with and without A23187 was measured. Treatment with A23187 rapidly (within 1-2 h) attenuated (p < 0.05) the time-dependent increase in basal and hCG-stimulated P production. Luteal P and PG production following treatment with the calcium ionophore, ionomycin, alone or in combination with additional CaCl2 was also monitored. Treatment with ionomycin (p < 0.01) and CaCl2 (p < 0.01) inhibited luteal P production. In contrast, treatment with ionomycin stimulated (p < 0.01) luteal PG production. To determine the effects of Ca2+ depletion on luteal function, P and PG production by cells incubated for 2 and 8 h in the absence and presence of the Ca2+-chelator EGTA was measured. Luteal production of both P and PG was inhibited by 8 h treatment with EGTA. In conclusion, elevations in [Ca2+]i differentially regulate PG and P production in the primate CL, leading to elevated PG and suppressed P production in CL collected in the mid-luteal phase. Since this pattern of luteal PG and P production is also evident late in the luteal phase, the phosphatidylinositol pathway may be involved in regression of the primate CL. In contrast to the selective alterations in luteal function following increases in [Ca2+]i, the depression of available calcium leads to suppression of both PG and P production. Thus, an optimal level of [Ca2+]i may be necessary for maximal luteal function.

INTRODUCTION

The role of the adenylate cyclase system in the regulation of the CL is well-documented [1-5]. However, the role of other second messenger systems in luteal regulation has not been extensively studied, particularly in the primate.

One alternative second messenger system that may be important in this regard is the phosphatidylinositol signaling system. Activation of this system initiates the hydrolysis of phosphatidylinositol-4,5-bis-phosphate to inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), and IP3 stimulates release of calcium from intracellular stores [7], thus resulting in a physiological response.

Recent evidence suggests that the phosphatidylinositol pathway could be involved in luteal regression. Treatment of nonprimate luteal cells with the calcium ionophore A23187, which increases intracellular free calcium, causes a decrease in LH-stimulated cAMP accumulation [8] and progesterone (P) production [8, 9]. Activation of this system by the luteolysin prostaglandin F2α (PGF2α) has been demonstrated in the CL of certain nonprimates [10-13].

The phosphatidylinositol pathway may also be involved in primate luteal regression. Treatment of human granulosa-luteal cells with PGF2α, a putative luteolysin, stimulates IP3 accumulation [14]. Calcium inhibits the responsiveness of adenylate cyclase in isolated luteal membranes from the primate [4, 15]. In addition, A23187 inhibits basal and hCG-stimulated P production by primate luteal cells during incubation for 8 h [16]. The response of the primate CL to calcium ionophores has not been investigated with incubation times of less than 8 h.

Similar to that of other species, the primate CL is composed of at least two different types of luteal cells [17, 18], which display differences in size, morphology, and function [17-19]. The response, in terms of decreases in intracellular free calcium concentrations ([Ca2+]i), to A23187 and PGF2α differs between large and small ovine luteal cells [12]. Since functional differences also exist within primate luteal subpopulations [17-19], important variations in such responses might exist among these cell types.

The objectives of the current study were (1) to further characterize the response of the primate CL to A23187, in terms of [Ca2+]i, and P production, and (2) to assess the effects of depletion, as well as elevating, available calcium on luteal P and prostaglandin (PG) production.

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MATERIALS AND METHODS

Treatment of Animals

The housing and general care of rhesus monkeys at The Ohio State University has been described previously [20]. Monkeys were checked daily for menses, and menstrual records were maintained (onset of menses = Day 1 of the menstrual cycle). Animals exhibiting regular cycles of approximately 28 days were used in the current study. Blood samples were collected daily by femoral venipuncture at 0800-1000 h from Day 8 of the menstrual cycle until luteectomy. The serum was stored at -20°C until assayed for hormones. On Day 14 of the cycle, serum concentrations of 17β-estradiol were determined by rapid RIA [21]. The day of the LH surge was estimated to occur 1 day before the precipitous decline in serum estrogen that followed the midcycle estrogen peak [22].

CL were collected via midventral laparotomy 8-10 days after the estimated day of the LH surge. Monkeys were anesthetized [20] before surgery with ketamine hydrochloride (Bristol Laboratories, Syracuse, NY; 10-15 mg/kg i.m.) supplemented with atropine sulfate (ESL Pharmaceuticals, Cherry Hill, NJ; 0.02 mg/kg i.m.) and sodium pentobarbital (Butler, Columbus, OH; 8-12 mg/kg, i.v.). The CL were excised from the ovaries by blunt dissection and immediately placed in Ham's F-10 medium (Sigma, St. Louis, MO) at 4°C for transport to the laboratory.

Preparation and Treatment of Tissue

Dispersed luteal cells were prepared separately from each CL by a combination of gentle mechanical agglutination and digestion with 0.25% collagenase (ESL Pharmaceuticals, Freehold, NJ) and 0.02% deoxyribonuclease (Sigma) [20]. Cell viability, as determined by trypan blue dye exclusion, was >95%. An aliquot of 200,000 cells was removed from luteal suspensions for the measurement of [Ca²⁺], by fura-2 fluorescence microscopy. The remainder of the luteal cells were then allowed to become loosely attached to a pyramidal shaker bath at concentrations of 50,000 cells/250 µl Ham's F-10 medium (0.299 mM Ca²⁺) at 37°C in an atmosphere of 95% O₂:5% CO₂.

To determine how quickly A23187 inhibits unstimulated luteal P production, cells were incubated for 1, 2, 4, and 8 h with and without A23187 (5 µM, n = 4 CL). To determine how quickly A23187 inhibits gonadotropin-stimulated progesterone production, this experiment was repeated in the presence of 500 ng/ml hCG (CR 127, 14 900 IU/mg, NIHDK, n = 3 CL). Brominated A23187 (+4-Br-A23187) was used in all experiments since it possesses less autofluorescence than the unbrominated A23187. Low autofluorescence is desirable to prevent interference with the accurate measurement of [Ca²⁺]. To examine the effects of ionomycin and CaCl₂ on the primate CL, luteal cells (n = 4 CL) were incubated for 4 h with no treatment, with ionomycin (0.1, 0.5, 1.0, 2.5, 5.0 µM), with CaCl₂ (0.5, 1.0, 2.0 mM), or with ionomycin (0.5, 1.0, 2.5 µM) + CaCl₂ (0.5, 1.0, 2.0 mM). Ionomycin is another calcium ionophore that is more specific for Ca²⁺ than A23187. To determine the effects of Ca²⁺ depletion on luteal function, cells were incubated for 2 and 8 h with a Ca²⁺-chelator, EGTA (1.0 mM), in the absence and presence of A23187 (5 µM). The viability of luteal cells after 8-h treatment with these doses of A23187, ionomycin, and EGTA was determined by trypan blue dye exclusion.

Cells and media were frozen at -20°C after incubation. Cells and media were separated by centrifugation at 160 x g prior to RIA. P concentrations were measured in all samples. Concentrations of a representative PG, PGE₂, were measured in the 8-h incubations of the Ca²⁺ depletion experiments and the ionomycin-CaCl₂ experiments. Production of PGE₂, PGE₁, and PGI₂ are influenced in the same manner by A23187 [16], therefore, PGE₂ was chosen as a representative PG since it is produced in the highest concentrations by the primate CL [16].

RIAs

RIAs were used to measure concentrations of P and PGE₂ in samples of F-10 medium incubated with dispersed cells [20]. Specific and proven antibodies for P (Dr. Gordon Niwender, Colorado State University) and PGE₂ (Dr. Harold Behrman, Yale University) were donated. Advanced Magnetics (Cambridge, MA) donated the PG standards. Radioactive P was purchased from New England Nuclear (Boston, MA), and radioactive PGE₂ was purchased from Amersham (Arlington Heights, IL).

The validation of these assays has been reported previously [20]. Crossreactivity of the PGE₂ antibody with 8 similar compounds is <0.1% (H. Behrman, personal communication). Exogenous treatments did not interfere with the RIAs. Coefficients of variation within and between assays were as follows: P, 8.96% and 11.68%; PGE₂, 7.22% and 14.46%.

Measurement of [Ca²⁺]

[Ca²⁺] was measured using fura-2 fluorescence microscopy of single cells as described by Li et al. [23]. A Zeiss IM microscope and a 63× PLAN NEOFLAR objective were used. Prior to microscopy, aliquots of 200,000 cells were incubated in 1 ml of F-10 medium with fura-2AM (6 µM) for 30 min at 37°C in a 95% O₂:5% CO₂ atmosphere. Cells were then washed and incubated for 60 additional min to allow for complete hydrolysis of the fura-2 ester. Luteal cells were then allowed to become loosely attached to a perfusion chamber. The ratio of emission (420-620 nm) of fura-2 at 348/380 nm (1A/2A) excitation was measured in small (17-21 µm) and large (23-28 µm) luteal cells (n = 5 CL; 5-11 cells/CL) at 0, 1, 2, 5, 10, and 15 min. The majority of cells within these two populations are steroidogenic [18]. A third population of smaller luteal cells was not studied; this population contains the majority (97% 4%) of nonste-
roidogenic cells [18]. Treatment with A23187 (5 μM) was begun 20–25 sec after initiation of measurement and was continued to 5 min. At the end of the treatment period, control buffer was perfused through the cell chamber; measurements then continued to 15 min. An increase in 1A/2A reflects an increase in [Ca²⁺].

Statistical Analyses

Analyses were performed using the following statistical programs: Statistical Analysis System (SAS Institute Inc., Cary, NC) and/or Number Cruncher Statistical System (JL Hinze, Kaysville, UT).

The response to A23187 over time was analyzed separately for those luteal cells treated with and without hCG. Three-way analyses of variance with individual CL as a block and A23187 and time as the main effects were used to compare P concentrations in these time trials. An interaction term of A23187 × time was also used in the model.

The effects of EGTA on P production at 2 and 8 h were analyzed separately using 3-way analyses of variance with a block on CL, EGTA and A23187 as the main effects. The model included EGTA × A23187 as the interaction term. The effects of EGTA on PGE₂ production at 8 h were analyzed using the same model.

The production of P and PGE₂ following treatment with ionomycin and CaCl₂ were analyzed separately using 3-way analyses of variance with a block on CL, Ionomycin and CaCl₂ were the main effects. The model included ionomycin × CaCl₂ as the interaction term. After a significant (p < 0.05) effect of ionomycin was demonstrated, comparisons between doses of ionomycin were made using the Student-Newman-Keuls test.

The response to A23187, in terms of [Ca²⁺], was analyzed using a nested design analysis of variance with cell type, individual cell (a block nested within cell type), and time as the main effects, and with cell type × time and individual cell × time as the interaction terms. The data were also analyzed separately for each cell type (large and small) using a 2-way analysis of variance with individual cell (block) and time as the main effects.

RESULTS

Treatment with A23187 at a dose that inhibits P production rapidly increased [Ca²⁺] in both cell types (Fig. 7). The increase in [Ca²⁺] was evident at 1 min (p < 0.01) in large cells and was maintained for the remaining observation period. In contrast, [Ca²⁺] did not increase until 5 min (p < 0.01) in small cells. However, once elevated, [Ca²⁺] was maintained at enhanced levels for the remaining period of observation, as it was in large cells. When compared in a single analysis, the pattern of increase in [Ca²⁺], following A23187 treatment did not significantly differ between cell types (p > 0.05 for cell type × time).

Treatment with A23187 attenuated (p < 0.05) the time-dependent increase in basal and hCG-stimulated P production (Fig. 8). This effect was evident within 1–2 h of incubation. Another calcium ionophore, ionomycin, also decreased (p < 0.01) P production. The effect of ionomycin was dose-dependent (Fig. 9A). Inhibition was significant at 0.5 μM and maximum at 2.5 μM CaCl₂; also inhibited (p < 0.01) P production (Fig. 9A). The effects of 0.5 mM and 2.0 mM CaCl₂ (data not shown) were not different from the effect of 1.0 mM CaCl₂. The pattern of response to ionomycin was not altered in the presence of CaCl₂ (p > 0.05 for interaction of ionomycin × CaCl₂).

In contrast to the inhibitory effect of P production, ionomycin (p < 0.01) stimulated PGE₂ production (Fig. 9B). The stimulation by ionomycin was dose-dependent. Stimulation was significant at 0.1 μM and maximum at 0.5 μM. These doses were much lower than those required for significant and maximum inhibition of P production. Although its effects were not significant (p = 0.12), CaCl₂ appeared to enhance the stimulatory effect of ionomycin on PG production (Fig. 9B). The effects of 0.5 mM and 2.0 mM CaCl₂ on PG production (data not shown) were similar to the effect of 1.0 mM CaCl₂.

The effects of EGTA on basal P and PGE₂ production are summarized in Table 2. At 2 h of incubation, EGTA (1.0 mM) did not significantly influence P production (p > 0.05). PGE₂ concentrations were not measured at 2 h since production is minimal at this time [20]. At 8 h of incubation, EGTA significantly inhibited both P (p < 0.05) and PGE₂ (p < 0.01) production. A23187 did not significantly alter the inhibition of P production by EGTA (p > 0.05) at 2 (data not shown) or 8 h (A23187 + EGTA, 18.8 ± 3.1 ng/ml; see Table 2 for control values). Similar inhibition was observed with A23187 alone (16.7 ± 5.5 ng/ml).

The viability of luteal cells following 8 h treatment with A23187 (87.8 ± 2.1%, x ± SE of % viable cells at 5 μM
A23187, ionomycin (86.9 ± 1.1%, 5 μM) or EGTA (85.8 ± 1.1%, 1 mM) was not significantly different (p > 0.05) from that of control (85.3 ± 1.6%). The lower doses of ionophores also did not alter viability (data not shown).

DISCUSSION

The results of this study indicate that doses of A23187 capable of suppressing luteal P production are also effective at increasing [Ca2+] in primate luteal cells. Similar doses of A23187 increase [Ca2+] in ovine luteal cells [12]. These data support the notion that A23187 has its effects on luteal P production by increasing [Ca2+], rather than via nonspecific effects. In addition, since A23187 appeared to increase [Ca2+], more quickly in large vs. small luteal cells, different cell types within the primate CL may respond differently to A23187 treatment. Differences between large and small ovine luteal cells, with regard to the changes in [Ca2+], in response to A23187, have also been observed. The increase in [Ca2+] is rapid and transient in small ovine luteal cells [12] and slow and sustained in large ovine luteal cells [12]. In monkey luteal cells, the increase is sustained in both cell types with a more rapid increase in large luteal cells.

Increases in [Ca2+] in response to PGE2, a physiological agent, have been observed in various nonprimate CL [10–13, 24]; however, in the ovine CL, this response is limited to large cells [12, 24]. The response of small and large primate luteal cells to PGE2, in terms of changes in [Ca2+], remains to be determined. However, stimulation of phosphatidylinositol breakdown by PGE2 has been demonstrated in human granulosal-luteal cells [14].

TABLE 2. Effects of EGTA (1 mM) on P and PGE2 production.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P (ng/ml)</th>
<th>PGE2 (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>2 h control (n = 6 CL)</td>
<td>27.6 ± 5.96</td>
<td>—</td>
</tr>
<tr>
<td>2 h EGTA (n = 6 CL)</td>
<td>21.8 ± 5.11</td>
<td>—</td>
</tr>
<tr>
<td>8 h control (n = 10 CL)</td>
<td>48.93 ± 11.89</td>
<td>1354.6 ± 186.5</td>
</tr>
<tr>
<td>8 h EGTA (n = 10 CL)</td>
<td>24.22 ± 4.01*</td>
<td>782.8 ± 67.2*</td>
</tr>
</tbody>
</table>

*Denotes significant difference from control within time (p < 0.05).
Inhibition of P production by A23187 treatment of primate luteal cells (mixed cell population) was previously demonstrated using an 8-h incubation [16]. The current study showed that the effect of A23187 on P production was rapid, occurring within 1–2 h. Thus, increases in [Ca^{2+}], which occur within 5 min after A23187 treatment, promote rapid interference with both basal and hCG-stimulated P production. Inhibition by A23187 was also evident within 2 h in rat and bovine luteal cells; however, whereas basal P production is inhibited by A23187 in large bovine luteal cells [9], only LH-stimulated P production is inhibited in rat [8] and small bovine [9] luteal cells.

This study revealed that treatment with ionomycin, an ionophore with more specificity for calcium, inhibited P production, in a dose-dependent manner, as has been shown for A23187 [16]. Similar doses of ionomycin inhibit LH-stimulated cAMP accumulation in the rat CL [25]. The addition of exogenous calcium to medium also inhibited P production and enhanced the inhibition by ionomycin. Although the effect of exogenously added calcium on [Ca^{2+}], was not directly measured, these findings support the notion that calcium ionophores suppress P production specifically through increases in [Ca^{2+}]. The effect of exogenous calcium was not dose-dependent in the range examined, perhaps because even the lowest dose (0.5 mM) added to the medium is manyfold greater than resting [Ca^{2+}], (~100 mM [Houmard et al., unpublished observations]). At lower concentrations, a dose-dependent response may be more evident. Inhibition by calcium of adenylate cyclase activity in isolated membranes from the primate CL [4, 15] provides further evidence for the inhibitory role of Ca^{2+} in luteal regulation in this species.

In contrast to the inhibitory effect on P production, exogenous calcium and calcium ionophores (ionomycin [current study], A23187 [16]) stimulated PG production. Maximal stimulation of PG production occurred at a much lower dose than maximal inhibition of P production, indicating that the synthetic machinery for PG production is more sensitive to elevations in [Ca^{2+}] than that for P synthesis. Thus, P and PG production are differentially regulated by [Ca^{2+}], with elevations leading to a shift toward increased PG production and decreased P production by the CL. A similar shift in PG and P production occurs in luteal cells collected from late luteal phase CL [16]. Perhaps elevations in [Ca^{2+}], as part of the phosphatidylinositol pathway, are involved in primate luteal regression.

The effects of elevations in [Ca^{2+}], in the primate CL may be mediated by various Ca^{2+}-dependent cellular proteins. The major targets of intracellular Ca^{2+} in other cell types are protein kinase C, calmodulin, calpains (proteases), phospholipases (C and A2), and ion channels (see [26] for review). The effects of activating these systems on primate luteal function have not been studied. However, inhibitory [27–29], as well as stimulatory [30, 31], effects of protein kinase C activation have been reported in CL from non-primate species. As mentioned above, increases in [Ca^{2+}], activate phospholipase A2 [32], the enzyme that releases arachidonic acid, the precursor, for PG production. In this way, increases in [Ca^{2+}], may lead to enhanced PG synthesis. Further studies are necessary to determine the precise mechanisms responsible for the modulation of luteal P and PG production by intracellular Ca^{2+}.

Whereas elevations in [Ca^{2+}], shift luteal function to increase PG production and decrease P production, depletion of extracellular Ca^{2+} with EGTA suppresses both PG and P production within 8 h in primate luteal cells. The inhibitory effect of EGTA on P production probably accounts for the inability of EGTA to overcome the inhibition by A23187. Treatment with EGTA also inhibits basal or agonist-stimulated P production in ovarian cells from other species [9, 33–37]. Depletion of extracellular Ca^{2+} with EGTA persists decreases [Ca^{2+}], as measured by indo-2 spectroscopy, in luteal cells [37]. Thus, some minimal level of calcium may be necessary for proper luteal function. Other studies have also reported that an optimal level of [Ca^{2+}], is necessary for maximal P production from large ovine [37] and bovine [9] luteal cells. As in primate luteal cells, either elevation or depletion of [Ca^{2+}], leads to decreased P production by large luteal cells from these species.

In conclusion, elevations in [Ca^{2+}], differentially regulate PG and P production in the primate CL, leading to elevated PG and suppressed P production in CL collected during the mid-luteal phase. Since this pattern of luteal P and PG production is also evident late in the luteal phase, these studies provide further evidence for a possible role of the phosphatidylinositol pathway in regression of the primate CL. In contrast to the selective alterations in luteal function following increases in [Ca^{2+}], depletion of available calcium leads to suppression of both PG and P production. Thus, an optimal level of [Ca^{2+}], may be necessary for maximal luteal function.

ACKNOWLEDGMENTS

The authors would like to thank Ann C. Omholt and Jennie L. Tausch for their scientific and technical contributions.

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CHAPTER III

Activation of the Phosphatidylinositol Pathway in the Primate Corpus Luteum by Prostaglandin $F_{2\alpha}$

Introduction

The role of the adenylate cyclase system in the regulation of the corpus luteum (CL) is well-documented. However, the role of other second messenger systems in luteal regulation has not been extensively studied, particularly in the primate. Activation of one of these pathways, the phosphatidylinositol system, initiates the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol triphosphate (IP$_3$) and diacylglycerol (DAG). DAG activates protein kinase C (1) and IP$_3$ stimulates release of calcium from intracellular stores (2). These events are important in the initiation of a number of physiological responses.

Recent evidence also suggests that the phosphatidylinositol pathway is involved in luteal regression. Treatment of nonprimate luteal cells with a calcium ionophore, prompting an increase in intracellular free calcium, causes a decrease in LH-stimulated cAMP accumulation (3) and progesterone production (3, 4). The
phosphatidylinositol pathway may also be involved in primate luteal regression. Calcium inhibits the responsiveness of adenylate cyclase in isolated luteal membranes from the primate (5, 6). In addition, treatment of primate luteal cells with various calcium ionophores inhibits basal and hCG-stimulated progesterone production (7, 8).

Activation of this system by a luteolysin, prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$), has been demonstrated in the CL of certain nonprimates (9-12). In the primate corpus luteum, activation of the phosphatidylinositol system and the dynamics of changes in intracellular free calcium have not been characterized. It is known, however, that treatment of human granulosal-luteal cells with PGF$_{2\alpha}$, a potential luteolysin in the primate, stimulates hydrolysis of phosphatidylinositol (13). Detailed studies of the activation of the phosphatidylinositol pathway during physiological responses of the primate CL are necessary to further implicate this pathway in primate luteal regression.

Similar to other species, the primate CL is composed of at least two different types of luteal cells (14, 15), which display differences in size, morphology, and function (14-16). The response to PGF$_{2\alpha}$, assessed by increases in intracellular free calcium concentrations ([Ca$^{2+}$]$_i$), differs between large and small ovine luteal cells (11). Since functional differences also exist within primate luteal subpopulations (14-16), important variations in activation
of the phosphatidylinositol pathway might exist among these cell types.

The objectives of the current study were (1) to assess the ability of PGF$_{2\alpha}$ to activate the phosphatidylinositol pathway by measuring changes in phosphatidylinositol hydrolysis and intracellular free calcium dynamics, (2) to determine if the responsiveness of the phosphatidylinositol system to PGF$_{2\alpha}$ depends on the stage of the luteal phase of the menstrual cycle, (3) to examine whether this responsiveness is altered during rescue of the CL by in vivo CG treatments which simulate early pregnancy, and (4) to determine whether this responsiveness differs with luteal cell type.

Materials and Methods

Treatment of Animals

The housing and general care of rhesus monkeys was as described previously (17). Briefly, monkeys were checked daily for menses, and menstrual records were maintained (onset of menses = Day 1 of the menstrual cycle). Animals exhibiting regular cycles of approximately 28 days were used in the current study. Blood samples were collected daily by femoral venipuncture at 0800-1000 h from Day 8 of the menstrual cycle until luteectomy. These samples were used to determine CL age and confirm normal luteal function. The serum was stored at -20 C until assayed for hormones. On
Day 14 of the cycle, serum concentrations of 17β-estradiol were determined by rapid RIA (18). The day of the LH surge was estimated to occur 1 day before the precipitous decline in serum estrogen that followed the midcycle estrogen peak (19).

A midventral laparotomy was performed for CL collection. Monkeys were anesthetized (17) before surgery with ketamine hydrochloride (Bristol Laboratories, Syracuse, NY; 10-15 mg/kg i.m.) supplemented with atropine sulfate (ESI Pharmaceuticals, Cherry Hill, NJ; 0.02 mg/kg, i.m.) and sodium pentobarbital (Butler, Columbus, OH; 8-12 mg/kg, i.v.). The CL were excised from the ovaries by blunt dissection and immediately placed in Ham's F-10 medium (Sigma, St. Louis, MO) at 4°C for transport to the laboratory.

CL were collected at various times after the estimated LH surge: d 4-5 (early luteal phase; n=4), d 8-9 (mid-luteal phase; n=4), and d 13-14 (late luteal phase; n=5). CL (n=5) were also collected following 1 day of in vivo hCG treatment (15 IU given at 0800 and 1700 h) which began during the mid-luteal phase (d 8-10 after the estimated LH surge). This represents the first day (d 1 of simulated early pregnancy) of a 10-day hCG treatment regimen which invokes pregnancy-like patterns of circulating CG, progesterone, and estradiol in nonpregnant rhesus monkeys (20). CL were collected one day after the initiation of in vivo hCG treatment. During
this period of simulated early pregnancy, the corpus luteum is in the initial stages of rescue, as evidenced by increasing levels of circulating progesterone in response to hCG (20).

**Preparation and Treatment of Tissue**

Dispersed luteal cells were prepared separately from each CL by a combination of gentle mechanical agitation and digestion with 0.25% collagenase (Worthington Biochemical, Freehold, NJ) and 0.02% deoxyribonuclease (Sigma) (17). Cell viability, as determined by trypan blue exclusion, was 90-95 %. An aliquot of 200,000 cells was removed from luteal suspensions for the measurement of $[Ca^{2+}]_i$ by fura-2 fluorescence photometry. The remainder of the luteal cells were washed twice in medium 199 (Sigma). A portion of these cells were used to monitor phosphatidylinositol hydrolysis in response to PGF$_{2\alpha}$ as described below. To monitor the effects of PGF$_{2\alpha}$ on progesterone production, suspensions of dispersed luteal cells (12,500 cells/250 µl medium) were incubated for 8h with increasing concentrations of PGF$_{2\alpha}$ (0, 5, 50, 500 ng/ml) in medium 199 at 37 C. Following incubation, the cells and medium were stored at -20 C. Prior to radioimmunoassay of the medium, cellular debris was removed by centrifugal separation (160 g).

**Measurement of phosphatidylinositol hydrolysis**

Phosphatidylinositol hydrolysis was monitored by quantifying the amount of labelled inositol phosphates
liberated (21) following treatment with PGF$\textsubscript{2}\alpha$. Phosphatidylinositol within the cellular membrane was labelled by incubating dispersed cells with 0.5 ml of $^3$H-myo-inositol (100 $\mu$Ci/ml; NEN Research Products, Boston, MA; specific activity = 15.4 Ci/mmol) in medium 199 at 37 C for 3h. The cells were then washed twice in fresh medium, incubated for another 15 min, and washed again to remove unincorporated $^3$H-myo-inositol. Labelled cells were aliquoted into culture tubes at a concentration of 150,000 cells/500 $\mu$l. Following a 5 minute pre-incubation with LiCl (10 mM), cells were incubated for 30 min with increasing concentrations of PGF$\textsubscript{2}\alpha$ (0, 5, 50, 500 ng/ml) in medium 199 with 10 mM LiCl. Lithium was included in the incubation medium to prevent the conversion of inositol phosphates to free inositol. The reaction was terminated by the addition of 170 $\mu$l of 10% (w/v) HClO$_4$. Following neutralization with KOH and saturated HEPES solution, tubes were placed in an ice bath for 30 min to precipitate KClO$_4$. The precipitate was pelleted by centrifugation at 2500 x g for 15 min, and the supernatant was stored at -20 C until chromatography.

To isolate inositol phosphates, samples were chromatographed as described by Bone et al. (21) on columns of AG 1-X8 resin (0.6 g; formate form) equilibrated with 10 ml of prep buffer (5 mM Na$_2$B$_4$O$_7$, 0.5 mM EDTA). Samples were thawed and applied to the column in 4 ml of prep buffer. $^3$H-labelled products were eluted by the sequential addition
of 10 ml H$_2$O; 20 ml 5 mM Na$_2$B$_4$O$_7$, 60 mM ammonium formate; 20 ml 5 mM Na$_2$B$_4$O$_7$, 150 mM ammonium formate (IP eluted); 20 ml 0.1 M formic acid, 0.4 M ammonium formate (IP$_2$ eluted); and 20 ml 0.1 M formic acid, 1 M ammonium formate (IP$_3$ eluted). The validity of this chromatography procedure was confirmed by following the elution of $^3$H-inositol and $^3$H-IP through the columns. As expected, $^3$H-inositol was eluted by the first two solutions and $^3$H-IP was eluted by the third solution. For all samples five ml fractions were collected from the columns. Two ml aliquots from these fractions were counted in Ultima Gold counting cocktail (Packard). The counting efficiency was not different among the various buffers. Since endogenous phosphatases convert IP$_3$ to IP$_2$ and IP$_2$ to IP, cpm recovered from these three fractions were combined to quantify the amount of labelled phosphatidylinositol hydrolyzed. The data were presented as fold-change from control to reflect PGF$_{2a}$-induced phosphatidylinositol hydrolysis.

**Measurement of [Ca$^{2+}$]$_i$**

[Ca$^{2+}$]$_i$ were measured using fura-2 fluorescence photometry of single cells (22). The use of this technique in primate luteal cells has been described previously (8). A Zeiss IM microscope and 63X PLAN NEOFLUAR objective were used. Prior to microscopy, aliquots of 200,000 cells were incubated in 1 ml of F-10 medium with fura-2AM (6 μM) for 30 min at 37 C in a 95% O$_2$:5% CO$_2$ atmosphere. Cells were then
washed and incubated for 60 additional min to allow for complete hydrolysis of the fura-2 ester. Luteal cells were then allowed to settle and become loosely attached to a perfusion chamber before perfusion began. The ratio of emission (420-620 nm) of fura-2 at 348/380 nm excitation was measured in small (17-21 μ) and large (23-28 μ) luteal cells (n=4-5 CL/group; 6-12 cells/CL) at 0, 1, 2, 5, 10, and 15 min. The majority of cells within these two populations are steroidogenic (15). A third population of smaller luteal cells was not studied. The latter population contains the majority (97.4%) of non-steroidogenic cells (15). Cells exhibiting morphological features characteristic of steroidogenic cells, such as a high content of lipid droplets, were selected for study. Treatment with PGF$_2$α (500 ng/ml) was begun 20-25 sec after initiation of measurement and was continued to 4 min. At the end of the treatment period, control buffer was perfused through the cell chamber; measurements then continued at intervals up to 15 min. The ratio of emission at 348/380 nm excitation was converted to [Ca$^{2+}$]$_i$ using the methods of Li et al. (22), as updated by Groden et al. (23).

**RIA**

RIA was used to measure concentrations of progesterone in samples of medium 199 incubated with dispersed cells, as validated previously (17). Specific and proven antibody for progesterone (Dr. Gordon Niswender, Colorado State
University) was donated. Tritiated progesterone was purchased from New England Nuclear (Boston, MA). Samples from individual CL were run within the same assay. The mean coefficient of variation within assays was 10.39%.

**Statistical Analyses**

Analyses were performed using the following statistical programs: Statistical Analysis System (SAS Institute Inc., Cary, NC) and/or Number Cruncher Statistical System (J.L. Hintze, Kaysville, UT).

The amounts of total labelled inositol phosphates accumulated in response to PGF$_{2\alpha}$ were compared using an analysis of variance. The main effects were treatment and reproductive stage with a block on CL. The interaction between treatment and stage was also included in the model. After a significant treatment*stage interaction was demonstrated, comparisons between doses of PGF$_{2\alpha}$ were made separately for each stage using the Student-Newman-Keuls test.

$[Ca^{2+}]_i$ following treatment with PGF$_{2\alpha}$ were analyzed using an analysis of variance with treatment, cell type (large or small) and reproductive stage as the main effects and individual cell as a block nested within cell type. The interaction between cell type and treatment, treatment and stage, and cell type and stage was also included in the analysis. The proportion of cells responding to PGF$_{2\alpha}$ with
an increase in $[\text{Ca}^{2+}]_i$ was compared across the different stages using a one-way analysis of variance.

The effects of PGF$_2\alpha$ on luteal progesterone production were analyzed using a three-way analysis of variance with a block on CL. Stage of the cycle or early pregnancy and treatment (PGF$_2\alpha$) were the main effects. The model included an interaction between treatment and stage. After a significant ($p<0.05$) effect of PGF$_2\alpha$ was demonstrated, comparisons between doses of PGF$_2\alpha$ were made using the Student-Newman-Keuls test.

**Results**

Total inositol phosphate accumulation in response to treatment with increasing concentrations of PGF$_2\alpha$ is shown in Figure 10. PGF$_2\alpha$ stimulated phosphatidylinositol hydrolysis in all groups. This stimulation was significant ($p<0.05$) in all but the mid-luteal phase group ($p=0.07$). However, the sensitivity to PGF$_2\alpha$ and the magnitude of response differed with the stage/state from which the CL were collected ($p<0.05$ for stage*treatment interaction). Lower doses of PGF$_2\alpha$ were capable of significantly stimulating IP accumulation in the late luteal phase and SEP groups as compared to the early and mid-luteal phase groups. CL treated with hCG *in vivo* (SEP group) displayed the most sensitivity and largest response to PGF$_2\alpha$. 
Most luteal cells responded to PGF₂α with an unequivocal increase in [Ca²⁺]ᵢ. The remaining cells did not display any change (p>0.10) in [Ca²⁺]ᵢ following PGF₂α treatment. The cells with a positive response to PGF₂α had a dramatic and transient increase in [Ca²⁺]ᵢ (p<0.01, Figure 11), occurring 50-80 sec after the initiation of PGF₂α treatment.

In the cells with a positive response, the pattern of [Ca²⁺]ᵢ following PGF₂α treatment differed with the stage/state from which the CL were collected (p<0.01). The increase in [Ca²⁺]ᵢ after PGF₂α treatment was greatest in the SEP group (Figure 11). The proportion of cells responding to PGF₂α with an increase in [Ca²⁺]ᵢ also varied with the stage/state from which the CL were collected (Table 3). This proportion was much lower in early luteal phase CL, as compared to CL collected mid or late LP or during in vivo hCG treatment.

The pattern of change in [Ca²⁺]ᵢ was similar in small and large luteal cells (Figure 11). Perhaps a small difference between the luteal cell types is evident in the recovery phase of the response in luteal cells collected during the mid- and late luteal phase. The proportion of positively responding cells did not differ between small and large luteal cells at any stage/state (Table 4). Since the effect of cell type on this proportion did not differ with
stage/state of the CL, Table 4 represents results pooled from all CL examined.

The in vitro response of luteal cells treated with PGF$_{2\alpha}$, as assessed by progesterone production, is depicted in Figure 12. Under the basal conditions examined, PGF$_{2\alpha}$ did not significantly (p>0.05) influence progesterone production by CL collected during the early, mid-, and late luteal phase. However, PGF$_{2\alpha}$ did significantly (p<0.05) inhibit progesterone production by CL collected after the in vivo hCG treatment of simulated early pregnancy (SEP).

Discussion

The results of this study demonstrate that PGF$_{2\alpha}$ is a potent activator of the phosphatidylinositol pathway in the primate CL. The PGF$_{2\alpha}$-induced increase in phosphatidylinositol hydrolysis observed in the current study is similar to that observed in the bovine CL (10) and in human granulosal-luteal cells (13). The pattern of [Ca$^{2+}$]$_i$ after PGF$_{2\alpha}$ is also similar to the pattern of increase in [Ca$^{2+}$]$_i$ following PGF$_{2\alpha}$ in luteal cells from other species (10-12, 24-26). Furthermore, it is known that increases in [Ca$^{2+}$]$_i$ induced by calcium ionophores inhibit luteal progesterone production in the primate (7, 8). Thus, the increase in [Ca$^{2+}$]$_i$ observed in the current study may mediate the inhibitory effects of PGF$_{2\alpha}$ on luteal
progesterone production which have been observed in other studies (27-30).

The activity of the phosphatidylinositol pathway has been compared in young and mature rat CL (31). With the exception of that work, the current study represents the first examination of the influence of CL age and in vivo gonadotropin treatment on activation of the phosphatidylinositol pathway by PGF$_2$$\alpha$. It is evident that the responsiveness of the primate CL to PGF$_2$$\alpha$, in terms of phosphatidylinositol hydrolysis and [Ca$^{2+}$]$_i$, varies throughout the luteal phase. In contrast to observations in the rat CL (31), the sensitivity of the primate CL to PGF$_2$$\alpha$, in terms of phosphatidylinositol hydrolysis, was low in early to mid-luteal phase as compared to late luteal phase. This is evidenced by the ability of lower doses of PGF$_2$$\alpha$ to significantly stimulate inositol phosphate accumulation in the late luteal phase CL as compared to early and mid-luteal phase CL. The proportion of luteal cells responding to PGF$_2$$\alpha$ with an increase in [Ca$^{2+}$]$_i$ was also low in the early luteal phase. Thus, the ability of PGF$_2$$\alpha$ to activate the phosphatidylinositol pathway, as assessed by two parameters, is minimal in the early luteal phase and increases by late luteal phase.

Other studies have shown that the luteolytic effects of PGF$_2$$\alpha$, in terms of progesterone production, are absent during the early luteal phase and appear during mid-late
luteal phase (27-29). Thus, the ability of PGF$_{2\alpha}$ to activate the phosphatidylinositol pathway develops throughout the luteal phase with the luteolytic effects of PGF$_{2\alpha}$. Previous studies from our laboratory have shown that PGF$_{2\alpha}$ is produced by the primate CL at high levels in both early and late luteal phase (7). Perhaps a combination of changes in production of PGF$_{2\alpha}$ and changes in the ability of PGF$_{2\alpha}$ to activate the phosphatidylinositol pathway ensure that luteolysis occurs at the appropriate time.

Interestingly, the largest increase in phosphatidylinositol hydrolysis and [Ca$^{2+}$]$_i$ following PGF$_{2\alpha}$ treatment occurred in the only group (d 1 SEP) to display an inhibitory effect of PGF$_{2\alpha}$ on progesterone production. Therefore, the ability to induce phosphatidylinositol hydrolysis may be linked to the ability to inhibit luteal progesterone production. Other studies have also shown that the inhibitory effects of PGF$_{2\alpha}$ on progesterone production by primate CL are most profound during gonadotropin stimulation (27). This phenomena also serves to explain the absence of the expected inhibitory effect of PGF$_{2\alpha}$ on progesterone production by mid and late luteal phase CL in the current study since gonadotropin was not present in the cell incubations.

Other studies have also reported an enhancement of PGF$_{2\alpha}$ activation of the phosphatidylinositol pathway by
gonadotropin. LH enhances PGF$_{2\alpha}$-induced increases in [Ca$^{2+}$]$_i$ in the bovine CL (12). Recently, Jacobs et al. (32) reported that in vitro treatment of ovine luteal cells with LH enhanced PGF$_{2\alpha}$-induced phosphatidylinositol hydrolysis. Studies are currently underway in our laboratory to examine the effect of in vitro gonadotropin on basal and PGF$_{2\alpha}$-induced activity of the phosphatidylinositol pathway.

The enhancement of luteolytic mechanisms by gonadotropin seems somewhat paradoxical since gonadotropin is responsible for rescue of the CL during primate early pregnancy. Although the phosphatidylinositol pathway appears responsive to PGF$_{2\alpha}$ during mid-luteal phase (current study), luteal production of PGF$_{2\alpha}$ is low during this stage (7). Increased production of luteal PGF$_{2\alpha}$ (7) and a high degree of responsiveness of the phosphatidylinositol pathway to PGF$_{2\alpha}$ (current study) during the late luteal phase may contribute to luteolysis in the menstrual cycle. During rescue of the CL by hCG in simulated early pregnancy, there is no late luteal phase increase in PGF$_{2\alpha}$ production by the CL (33). Similarly, CL treated with hCG in vivo release lower amounts of PGF$_{2\alpha}$ in vitro than age-matched CL in the late luteal phase (34). Thus, although gonadotropin enhances the ability of exogenous PGF$_{2\alpha}$ to activate the phosphatidylinositol pathway, suppressed levels of endogenous PGF$_{2\alpha}$ during early pregnancy may allow for rescue of the CL. The hypothesis
that CG acts to rescue the CL via a mechanism other than direct inhibition of the luteolytic actions of PGF$_{2\alpha}$ is supported by the observation that exogenous PGF$_{2\alpha}$ analogue can inhibit luteal function during rescue of the CL by hCG (35).

Differences between small and large luteal cells were not evident in either the pattern of increase in [Ca$^{2+}$]$_i$ after PGF$_{2\alpha}$ or the proportion of positively responding cells. Similarly, PGF$_{2\alpha}$ increases [Ca$^{2+}$]$_i$ in small (36) and large (12) luteal cells from the bovine CL. In contrast, large, but not small, cells from the ovine CL responded to PGF$_{2\alpha}$ with an increase in [Ca$^{2+}$]$_i$ (11). Thus, the response of different luteal cell types to PGF$_{2\alpha}$, as assessed by activation of the phosphatidylinositol pathway, may be species-dependent.

In conclusion, this study demonstrates that PGF$_{2\alpha}$ activates the phosphatidylinositol pathway in the primate CL. Responsiveness of the PI pathway to PGF$_{2\alpha}$ is low during the early luteal phase, increases by late luteal phase and is enhanced by in vivo gonadotropin treatment. This study, therefore, provides evidence that the inhibitory effects of PGF$_{2\alpha}$ on progesterone production are associated with the prompt activation of the phosphatidylinositol pathway.
Figure 10. Fold change in total inositol phosphate (IPs) accumulation following treatment of primate luteal cells (n=4-5 CL/group) with prostaglandin (PG) F$_2$α. Luteal cells (150,000 cells/500 μl) collected from various stages of the luteal phase (LP) and after 1 day of simulated early pregnancy (SEP) were incubated in the absence and presence of increasing doses of PGF$_2$α for 30 min. An asterisk indicates significant difference (p<0.05) from control within a group.
Figure 11. Intracellular free calcium concentrations ([Ca$^{2+}$]$_i$) after PGF$_{2\alpha}$ treatment in luteal cells collected from early (n=4 CL), mid (n=4 CL), and late (n=5 CL) luteal phase (LP) and following 1 day of in vivo hCG treatment (d 1 of simulated early pregnancy [SEP d1]; n=5 CL). Patterns of [Ca$^{2+}$]$_i$ are depicted separately for small (17-21 µ) and large (23-28 µ) luteal cells. Roughly equivalent numbers of small and large cells were examined. The data were standardized to the onset of the peak, which began 50-80 sec after the initiation of PGF$_{2\alpha}$ treatment. Only data from positively responding luteal cells (n=76) were depicted. See Tables 3 and 4 for data on the proportions of cells that responded positively to PGF$_{2\alpha}$. The increase in [Ca$^{2+}$]$_i$ after PGF$_{2\alpha}$ was significant (p<0.01) for positively responding cells in all groups.
Figure 12. Progesterone production by luteal cells (n=4-5 CL/group) collected from various stages of the luteal phase (LP) and during simulated early pregnancy (SEP, d 1). Luteal cells (12,500 cells/250 μl) were incubated in the absence and presence of increasing doses of PGF₂α for 8 h. An asterisk indicates a significant difference (p<0.05) from control within group. The standard error of the means from the model used to analyze the data was 0.34.
Table 3. Proportion (mean±SE) of Luteal Cells (n=18 CL, 6-12 cells/CL) Responding to PGF$_{2\alpha}$ with an Increase in Intracellular Free Calcium at Various Stages/States of the Luteal Phase.

<table>
<thead>
<tr>
<th>Stage/State</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Luteal Phase</td>
<td>0.18±.03$^a$</td>
</tr>
<tr>
<td>Mid-Luteal Phase</td>
<td>0.72±.03$^b$</td>
</tr>
<tr>
<td>Late Luteal Phase</td>
<td>0.71±.16$^b$</td>
</tr>
<tr>
<td>Simulated Early Pregnancy (d 1)</td>
<td>0.65±.14$^b$</td>
</tr>
</tbody>
</table>

Means with different letter designations displayed significant differences in proportion (p<0.05).
Table 4. Proportion (mean±SE) of Small and Large Luteal Cells (n=18 CL, 6-12 cells/CL) Responding to PGF$_{2\alpha}$ with an Increase in Intracellular Free Calcium.

<table>
<thead>
<tr>
<th>Cell Type*</th>
<th>Proportion**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>0.61±.08</td>
</tr>
<tr>
<td>Large</td>
<td>0.56±.09</td>
</tr>
</tbody>
</table>

*No significant effect (p>0.05) on proportion of positive responding cells.

**Equal numbers of small and large cells were examined in CL of various stages/states.
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CHAPTER IV

The Effects of Gonadotropin on Basal and PGF$_{2\alpha}$-Induced Activity of the Phosphatidylinositol Pathway in the Primate Corpus Luteum

Introduction

The role of the adenylate cyclase system in the regulation of the corpus luteum (CL) is well-documented. Recently, it has become clear that other second messenger systems are active in the CL and may regulate luteal function. One such system is the phosphatidylinositol signaling pathway. Activation of this pathway initiates the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol triphosphate (IP$_3$) and diacylglycerol (DAG). DAG activates protein kinase C (1) and IP$_3$ stimulates release of calcium from intracellular stores (2). Such events are important in the initiation of a number of physiological responses.

Recent evidence suggests that the phosphatidylinositol pathway may be involved in luteal regression of several species, including the primate. Calcium inhibits the responsiveness of adenylate cyclase in isolated luteal membranes from the primate (3, 4). In addition, treatment
of primate luteal cells with various calcium ionophores inhibits basal and hCG-stimulated progesterone production (5, 6). Thus, agents that mimic the activity of the phosphatidylinositol pathway elicit inhibitory responses in the primate CL. Interestingly, a potential luteolysin in the primate, PGF$_{2\alpha}$, activates the phosphatidylinositol pathway in monkey luteal cells (7) and human granulosa-luteal cells (8). Activation by PGF$_{2\alpha}$ is characterized by large increases in phosphatidylinositol hydrolysis (7, 8) and a distinct rise in intracellular free calcium concentrations ([Ca$^{2+}$]$_i$) (7).

The effects of luteotropic agents, such as LH or hCG, on the activation of the phosphatidylinositol system and the dynamics of changes in [Ca$^{2+}$]$_i$ in the primate CL have not been characterized. Furthermore, gonadotropin appears to activate the phosphatidylinositol pathway in luteal cells of some species (9-12), but not others (13-16). It has also been reported that hCG does not alter phosphatidylinositol hydrolysis in human granulosa-luteal cells (8). Further studies of the effects of gonadotropin on phosphatidylinositol hydrolysis, as well as changes in [Ca$^{2+}$]$_i$, are necessary to increase our understanding of the role of the phosphatidylinositol pathway in primate luteal regulation.

Similar to other species, the primate CL is composed of at least two different types of luteal cells (17, 18), which
display differences in size, morphology, and function (17-19). Since the increase in $[\text{Ca}^{2+}]_i$ induced by gonadotropin differs between large and small cells of the bovine CL (11), important variations in activation of the phosphatidylinositol pathway might also exist among these cell types in the primate CL.

If luteolysis in the primate results from activation of the phosphatidylinositol pathway by PGF$_{2\alpha}$, as suggested above, it is important to determine how CG overcomes the luteolytic mechanism to rescue the CL during early pregnancy. It is possible that CG interferes with the ability of luteolysins to activate the phosphatidylinositol pathway.

The objective of the current study was to investigate the effects of gonadotropin on basal (Expt. 1) and PGF$_{2\alpha}$-stimulated (Expt. 2) activity of the phosphatidylinositol pathway in the primate CL. In each of these experiments, we sought (1) to determine whether the responses of the phosphatidylinositol system to gonadotropin change throughout the luteal phase of the menstrual cycle, (2) to examine whether the in vitro effects of gonadotropin are altered during rescue of the CL by in vivo CG treatments which simulate early pregnancy, and (3) to determine whether these responses differ with luteal cell type.

Our results indicate that, in the primate CL, luteotropins are less potent activators of the
phosphatidylinositol pathway than luteolytic agents. Thus, this further supports the hypothesis that luteolysis in the primate may result from activation of the phosphatidylinositol pathway. In addition, in vitro hCG did not alter PGF$_{2\alpha}$-induced activity of the phosphatidylinositol pathway. Therefore, rescue of the CL during early pregnancy does not appear to result from direct interference of CG with the ability of luteolysins to activate this pathway.

**Materials and Methods**

**Treatments of Animals**

The housing and general care of rhesus monkeys was as described previously (20). Briefly, monkeys were checked daily for menses, and menstrual records were maintained (onset of menses = Day 1 of the menstrual cycle). Animals exhibiting regular cycles of approximately 28 days were used in the current study. Blood samples were collected daily by femoral venipuncture at 0800-1000 h from Day 8 of the menstrual cycle until luteectomy. These samples were used to determine CL age and confirm normal luteal function. The serum was stored at -20 C until assayed for hormones. On Day 14 of the cycle, serum concentrations of 17β-estradiol were determined by rapid RIA (21). The day of the LH surge was estimated to occur 1 day before the precipitous decline.
in serum estrogen that followed the midcycle estrogen peak (22).

A midventral laparotomy was performed for CL collection. Monkeys were anesthetized (20) before surgery with ketamine hydrochloride (Bristol Laboratories, Syracuse, NY; 10-15 mg/kg i.m.) supplemented with atropine sulfate (ESI Pharmaceuticals, Cherry Hill, NJ; 0.02 mg/kg, i.m.) and sodium pentobarbital (Butler, Columbus, OH; 8-12 mg/kg, i.v.). The CL were excised from the ovaries by blunt dissection and immediately placed in Ham's F-10 medium (Sigma, St. Louis, MO) at 4°C for transport to the laboratory.

CL (n=11, Expt. 1; n=9, Expt. 2) were collected at various times after the estimated LH surge: d 4-5 (early luteal phase), d 8-9 (mid-luteal phase), and d 13-14 (late luteal phase). CL were also collected following 1 day of in vivo hCG treatment (15 IU given at 0800 and 1700 h) which began during the mid-luteal phase (d 8-10 after the estimated LH surge). This represents the first day (d 1 of simulated early pregnancy) of a 10-day hCG treatment regimen which invokes pregnancy-like patterns of circulating CG, progesterone, and estradiol in nonpregnant rhesus monkeys (23). CL were collected one day after the initiation of in vivo hCG treatment. During this period of simulated early pregnancy, the corpus luteum is in the initial stages of
rescue, as evidenced by increasing levels of circulating progesterone in response to hCG (23).

**Preparation and Treatment of Tissue**

Dispersed luteal cells were prepared separately from each CL by a combination of gentle mechanical agitation and digestion with 0.25% collagenase (Worthington Biochemical, Freehold, NJ) and 0.02% deoxyribonuclease (Sigma) (20). Cell viability, as determined by trypan blue exclusion, was 90-95%. An aliquot of 200,000-400,000 cells was removed from luteal suspensions for the measurement of $[	ext{Ca}^{2+}]_i$ by fura-2 fluorescence photometry. The remainder of the luteal cells were washed twice in medium 199 (Sigma). A portion of these cells were used to monitor phosphatidylinositol hydrolysis in response to hCG in the absence (Expt. 1) and presence (Expt. 2) of PGF$_2\alpha$ as described below. To monitor the effects of hCG on basal progesterone production (Expt. 1), suspensions of dispersed luteal cells (12,500 cells/250 µl medium) were incubated for 8h with increasing concentrations of hCG (0, 0.05, 5, 500 ng/ml) in medium 199 at 37 C. The combined effects of hCG and PGF$_2\alpha$ on progesterone production (Expt. 2) were examined by incubating luteal cells as described above with increasing concentrations of PGF$_2\alpha$ (0, 50, 500 ng/ml) in the absence and presence of hCG (500 ng/ml). Following incubation, the cells and medium were stored at -20 C. Prior to
radioimmunoassay of the medium, cellular debris was removed by centrifugal separation (160 g).

**Measurement of phosphatidylinositol hydrolysis**

Phosphatidylinositol hydrolysis was monitored by quantifying the amount of labelled inositol phosphates liberated (24) following treatment with hCG (±PGF$_{2\alpha}$). Phosphatidylinositol within the cellular membrane was labelled by incubating dispersed cells with 0.5 ml of $^3$H-myo-inositol (100 μCi/ml; NEN Research Products, Boston, MA; specific activity = 15.4 Ci/mmol) in medium 199 at 37 C for 3h. The cells were then washed twice in fresh medium, incubated for another 15 min, and washed again to remove unincorporated $^3$H-myo-inositol. Labelled cells were aliquoted into culture tubes at a concentration of 150,000 cells/500 μl. Following a 5 minute pre-incubation with LiCl (10 mM), cells were incubated for 30 min with increasing concentrations of treatment in medium 199 with 10 mM LiCl. In Expt. 1, treatment consisted of increasing concentrations of hCG (0, 0.05, 5, 500 ng/ml). In expt. 2, treatment consisted of increasing concentrations of PGF$_{2\alpha}$ (0, 50, 500 ng/ml) in the absence and presence of hCG (500 ng/ml). Lithium was included in the incubation medium to prevent the conversion of inositol phosphates to free inositol. The reaction was terminated by the addition of 170 μl of 10% (w/v) HClO$_4$. Following neutralization with KOH and
saturated HEPES solution, tubes were placed in an ice bath for 30 min to precipitate KClO$_4$. The precipitate was pelleted by centrifugation at 2500 x g for 15 min, and the supernatant was stored at -20 C until chromatography.

To isolate inositol phosphates, samples were chromatographed as described by Bone et al. (24) on columns of AG 1-X8 resin (0.6 g; formate form) equilibrated with 10 ml of prep buffer (5 mM Na$_2$B$_4$O$_7$, 0.5 mM EDTA). Samples were thawed and applied to the column in 4 ml of prep buffer.

$^3$H-labelled products were eluted by the sequential addition of 10 ml H$_2$O; 20 ml 5 mM Na$_2$B$_4$O$_7$, 60 mM ammonium formate; 20 ml 5 mM Na$_2$B$_4$O$_7$, 150 mM ammonium formate (IP eluted); 20 ml 0.1 M formic acid, 0.4 M ammonium formate (IP$_2$ eluted); and 20 ml 0.1 M formic acid, 1 M ammonium formate (IP$_3$ eluted). The validity of this chromatography procedure was confirmed by following the elution of $^3$H-inositol and $^3$H-IP through the columns. As expected, $^3$H-inositol was eluted by the first two solutions and $^3$H-IP was eluted by the third solution. For all samples five ml fractions were collected from the columns. Two ml aliquots from these fractions were counted in Ultima Gold counting cocktail (Packard). The counting efficiency was not different among the various buffers. Since endogenous phosphatases convert IP$_3$ to IP$_2$ and IP$_2$ to IP, cpm recovered from these three fractions were combined to quantify the amount of labelled phosphatidylinositol hydrolyzed. The data were presented as
fold-change from control to reflect agonist-induced phosphatidylinositol hydrolysis.

**Measurement of $[\text{Ca}^{2+}]_i$**

$[\text{Ca}^{2+}]_i$ was measured using fura-2 fluorescence photometry of single cells (25). The use of this technique in primate luteal cells has been described previously (6, 7). Briefly, a Zeiss IM microscope with a 63X PLAN NEOFLUAR objective was used. Prior to microscopy, aliquots of 200,000-400,000 cells were incubated in 1 ml of F-10 medium with fura-2AM (5-6 μM) for 30 min at 37°C in a 95% O₂:5% CO₂ atmosphere. Cells were then washed and incubated for 60 additional min to allow for complete hydrolysis of the fura-2 ester. Luteal cells were then allowed to settle and become loosely attached to a perfusion chamber before perfusion began. The ratio of emission (420-620 nm) of fura-2 at 348/380 nm excitation was measured in small (17-21 μ) and large (23-28 μ) luteal cells (n=6-12 cells/CL) from 0-4 min. The majority of cells within these two populations are steroidogenic (18). A third population of smaller luteal cells was not studied. The latter population contains the majority (97.4%) of non-steroidogenic cells (18). Cells exhibiting morphological features characteristic of steroidogenic cells, such as a high content of lipid droplets, were selected for study. In Expt. 1, cells from 6 of the 11 CL were treated with hCG (500 ng/ml) beginning 20-25 sec after initiation of
measurement. HCG treatment was continued to 4 min. To examine the effect of hCG on PGF$_{2\alpha}$-induced increases in [Ca$^{2+}$]$_i$ (Expt. 2, n=9 CL), the responses to PGF$_{2\alpha}$ (500 ng/ml) alone and to PGF$_{2\alpha}$ in combination with hCG (500 ng/ml) were measured in separate treatment periods in each luteal cell. The first treatment period began 20-25 sec after initiation of measurement and was continued for 4 min. At the end of this first period, control buffer was perfused through the cell chamber for 15 min to remove the first treatment and allow the [Ca$^{2+}$]$_i$ within the luteal cell to return to resting levels. The second treatment period began 20-25 sec after reinitiation of measurement and was continued for 4 min. The order of treatments (PGF$_{2\alpha}$ alone or PGF$_{2\alpha}$+hCG) for each cell was randomized. The ratio of emission at 348/380 nm excitation was converted to [Ca$^{2+}$]$_i$ using the methods of Li et al. (25), as updated by Groden et al. (26).

**RIA**

RIA was used to measure concentrations of progesterone in samples of medium 199 incubated with dispersed cells, as validated previously (20). Specific and proven antibody for progesterone (Dr. Gordon Niswender, Colorado State University) was donated. Tritiated progesterone was purchased from New England Nuclear (Boston, MA). Samples from individual CL were run within the same assay. The mean coefficient of variation within assays was 11.2%.
Statistical Analyses and Data Presentation

Analyses were performed using the following statistical programs: Statistical Analysis System (SAS Institute Inc., Cary, NC) and/or NCSS (J.L. Hintze, Kaysville, UT).

Since the effects of hCG (±PGF$_{2\alpha}$) on phosphatidylinositol hydrolysis or [Ca$^{2+}$]$_i$ did not differ with the stage/state from which the CL were collected, data from CL of the various stages/states were pooled for analysis and presentation.

The effects of hCG on accumulation of labelled inositol phosphates and luteal progesterone production (Expt. 1) were compared, in separate analyses, using analyses of variance. The main effect was treatment with a block on CL. Analyses of variance were also used to compare these responses to hCG in the absence and presence of PGF$_{2\alpha}$ (Expt. 2). The main effects of these analyses were hCG treatment and PGF$_{2\alpha}$ treatment with a block on CL. An hCG*PGF$_{2\alpha}$ interaction term was also included in the models.

[Ca$^{2+}$]$_i$ alterations following treatment with hCG (Expt. 1) were analyzed using an analysis of variance with hCG treatment and cell type (large or small) as the main effects and individual cell as a block nested within cell type. The interaction between cell type and hCG treatment was also included in the analysis. To examine the effect of hCG on PGF$_{2\alpha}$-induced [Ca$^{2+}$]$_i$ changes (Expt. 2), a similar analysis of variance was used. The main effects were PGF$_{2\alpha}$
treatment, hCG treatment, cell type, and individual cell as a block nested within cell type. The interaction terms of PGF$_{2\alpha}$*hCG and cell type*PGF$_{2\alpha}$*hCG were included in the model.

Results

As mentioned previously, the response to hCG treatment, as determined by phosphatidylinositol hydrolysis and alterations in [Ca$^{2+}$]$_i$, was independent of stage/state of the CL in both experiments. Therefore, the data have been pooled from all stages in CL development, as illustrated below.

Expt. 1

Total inositol phosphate accumulation in primate luteal cells was not altered by treatment with increasing concentrations of hCG (Figure 13). However, approximately 70% of the luteal cells examined displayed a gradual, yet sustained, increase in baseline [Ca$^{2+}$]$_i$ following hCG treatment (p<0.05; Figure 14). The pattern of increase in [Ca$^{2+}$]$_i$ after hCG treatment and the proportion of positively responding cells did not differ with the stage/state from which the CL were collected or the luteal cell type examined.

Despite the meager responses of the phosphatidylinositol pathway to hCG, luteal progesterone
production was significantly increased (p<0.05) by the two highest concentrations of hCG examined (Figure 15).

**Expt. 2**

Figure 16 depicts inositol phosphate accumulation in response to increasing concentrations of PGF$_{2\alpha}$ in the absence and presence of hCG. As we have shown previously (7), inositol phosphate accumulation is significantly increased (p<0.05) by these concentrations of PGF$_{2\alpha}$. HCG did not alter this response to PGF$_{2\alpha}$ (p>0.05).

[Ca$^{2+}$]$_i$ responses following PGF$_{2\alpha}$ treatment alone and in combination with hCG were similar (Figure 17). As we have observed in a previous study (7), PGF$_{2\alpha}$ rapidly induced a large, yet transient, increase (p<0.01) in [Ca$^{2+}$]$_i$ in most luteal cells. Neither the pattern of response to PGF$_{2\alpha}$ nor the proportion of luteal cells responding to PGF$_{2\alpha}$ with an increase in [Ca$^{2+}$]$_i$ were altered by in vitro hCG (p>0.10). The time to the onset of the peak in [Ca$^{2+}$]$_i$ was also similar in the absence (27.0 ± 1.9 sec) and presence (27.9 ± 2.0 sec) of hCG.

The effects of PGF$_{2\alpha}$ on basal and hCG-stimulated progesterone production are shown in Figure 18. PGF$_{2\alpha}$ did not significantly alter luteal progesterone production under basal conditions. As expected, hCG significantly stimulated (p<0.05) progesterone production. PGF$_{2\alpha}$ inhibited (p<0.05) the stimulation by hCG.


Discussion

This study demonstrates that, in the primate CL, gonadotropin is a less potent activator of the phosphatidylinositol pathway than luteolytic agents. HCG did not markedly influence phosphatidylinositol hydrolysis and produced only slight elevations in $[Ca^{2+}]_i$ (~100 nM). The concentrations of hCG tested were clearly biologically active since they stimulated progesterone production. These meager effects of hCG on the phosphatidylinositol pathway contrast with the effects of PGF$_{2\alpha}$, a potential luteolysin in the primate (27-29). PGF$_{2\alpha}$ stimulates phosphatidylinositol hydrolysis and produces large increases in $[Ca^{2+}]_i$ (~1000 nM) (7, current study). Other agents that increase $[Ca^{2+}]_i$ (eg. calcium ionophores) suppress basal and hCG-stimulated progesterone production in primate luteal cells (5, 6). These observations, in combination with the current findings that luteolysins are more potent activators of the PI pathway than luteotropins, support the hypothesis that the phosphatidylinositol pathway plays a role in primate luteal regression.

Davis et al. (8) reported that gonadotropin also does not alter phosphatidylinositol hydrolysis in human granulosal-luteal cells. Studies in the rat (13, 14) and sheep (15, 16) CL have also failed to demonstrate an effect of gonadotropin on phosphatidylinositol hydrolysis and/or $[Ca^{2+}]_i$. However, gonadotropin does activate the
phosphatidylinositol pathway, as evidenced by increases in phosphatidylinositol hydrolysis or increases in $[\text{Ca}^{2+}]_i$, in luteal cells from the pig (30) and cow (9-12) and in granulosal cells from the pig (31) and rat (32). In addition, the murine LH receptor has recently been shown to be coupled to both the adenylate cyclase pathway and the phosphatidylinositol system (33). Differences in the structure of the murine and primate LH receptor (33) may account for the differential effects of gonadotropin on the phosphatidylinositol pathway in these species. Therefore, it appears that the coupling of gonadotropin to this pathway is species-dependent.

Although hCG is not a potent activator of the phosphatidylinositol pathway in the primate CL, hCG does induce a slight increase in baseline $[\text{Ca}^{2+}]_i$. The role of this change in $[\text{Ca}^{2+}]_i$ in the action of gonadotropin on the primate luteal cell requires further study. However, recent evidence suggests that an optimal level of $[\text{Ca}^{2+}]_i$ may exist for maximal progesterone production by the CL. This hypothesis comes from studies which have demonstrated that decreases, as well as increases, in $[\text{Ca}^{2+}]_i$ can suppress luteal progesterone production (6, 34, 35). Perhaps, the small elevation in $[\text{Ca}^{2+}]_i$ induced by hCG moves the intracellular calcium level toward that optimal level.

The mechanism responsible for the increase in $[\text{Ca}^{2+}]_i$ induced by hCG is also unclear. The results of the current
study failed to show any significant effect of hCG on phosphatidylinositol hydrolysis. Thus, either the increase in $[\text{Ca}^{2+}]_i$ is independent of IP$_3$ production or the levels of inositol phosphates generated by hCG are too small to be detected with the techniques used. That the hCG-induced increase in $[\text{Ca}^{2+}]_i$ is independent of IP$_3$ is supported by the observation that the pattern of increase in $[\text{Ca}^{2+}]_i$ following hCG is much different from that following PGF$_{2\alpha}$, an effect which is associated with large increases in inositol phosphate accumulation (7). Alila et al. (11) have provided evidence that the increase in $[\text{Ca}^{2+}]_i$ induced by LH in large bovine luteal cells is mediated by voltage-independent calcium channels. Interestingly, the pattern and magnitude of increase in $[\text{Ca}^{2+}]_i$ in these cells is very similar to that observed in the primate luteal cells examined in the current study.

The current study also demonstrates that in vitro gonadotropin does not alter PGF$_{2\alpha}$-induced activity of the phosphatidylinositol pathway in primate CL collected at various stages of the luteal phase and during simulated early pregnancy. Similar results were obtained with human granulosa-luteal cells (8). However, studies in other species have reported an enhancement of PGF$_{2\alpha}$ activation of the phosphatidylinositol pathway by gonadotropin (11, 16). Similarly, previous studies from our laboratory have shown that hCG administered in vivo to simulate early pregnancy
enhances PGF$_{2\alpha}$-stimulated phosphatidylinositol hydrolysis and [Ca$^{2+}$]$_i$ changes (7). The mechanism by which in vivo hCG enhances these responses remains unknown. However, the current study suggests that this effect cannot be mimicked by acute treatment with hCG in vitro in primate luteal cells. Perhaps the effect of hCG on PGF$_{2\alpha}$-induced activity of the phosphatidylinositol pathway requires chronic treatment or is dependent on in vivo factors not present in the in vitro system.

Since gonadotropin is responsible for rescue of the CL during primate early pregnancy, and primate luteal regression may involve activation of the phosphatidylinositol pathway by PGF$_{2\alpha}$, it appears somewhat incongruous that these luteolytic mechanisms are unaffected (in vitro hCG) or even enhanced (in vivo hCG) by hCG. However, with a few exceptions (36, 37), most studies in the primate have demonstrated that hCG also does not prevent the luteolytic effects of PGF$_{2\alpha}$ on luteal progesterone and/or cAMP production in vitro (current study, 27, 38, 39) or in vivo (40, 41). It is possible that chorionic gonadotropin prevents the production of the luteolytic substance(s) rather than directly inhibiting the action of the luteolysin(s) on the CL. Previous studies have suggested that increased production of luteal PGF$_{2\alpha}$ (5) and a high degree of responsiveness of the phosphatidylinositol pathway to PGF$_{2\alpha}$ (7) during the late luteal phase may contribute to
luteolysis in the menstrual cycle. During rescue of the CL by hCG in simulated early pregnancy, there is no late luteal phase increase in PGF$_{2\alpha}$ production by the CL (42). Similarly, CL treated with hCG in vivo release lower amounts of PGF$_{2\alpha}$ in vitro than age-matched CL in the late luteal phase (43). Thus, although gonadotropin does not prevent exogenous PGF$_{2\alpha}$ from activating the phosphatidylinositol pathway, suppressed production of endogenous PGF$_{2\alpha}$ during early pregnancy may allow for rescue of the CL.

In conclusion, this study demonstrates that luteolytic agents, such as PGF$_{2\alpha}$, are more potent activators of the phosphatidylinositol pathway than luteotropins, such as hCG. This further supports the hypothesis that the phosphatidylinositol pathway is involved in primate luteal regression. This study also shows that hCG does not acutely alter the responsiveness of the phosphatidylinositol pathway to PGF$_{2\alpha}$. This suggests that CG may rescue the CL of early pregnancy via a mechanism other than direct inhibition of the luteolytic actions of PGF$_{2\alpha}$. 
Figure 13. Fold change in total inositol phosphate (IPs) accumulation following treatment of primate luteal cells with hCG. Luteal cells (150,000 cells/500 μl) were incubated in the absence and presence of increasing doses of hCG for 30 min.
Figure 14. Intracellular free calcium concentrations ([Ca\(^{2+}\)]\(_i\)) in luteal cells (n=6 CL) following hCG treatment (500 ng/ml). Treatment began 25 sec after the initiation of measurement. Only data from positively responding luteal cells (n= 21) were depicted. The increase in [Ca\(^{2+}\)]\(_i\) after hCG was significant (p<0.01) for positively responding cells. The standard error of the means from the model used to analyze the data was 6.38.
Figure 15. Progesterone production by luteal cells (12,500 cells/250 μl) incubated in the absence and presence of increasing doses of hCG for 8 h. An asterisk indicates a significant difference (p<0.05) from control. The standard error of the means from the model used to analyze the data was 0.75.
Figure 16. Fold change in total inositol phosphate accumulation following treatment of primate luteal cells with prostaglandin (PG) F$_{2\alpha}$ in the absence and presence of hCG (500 ng/ml). Luteal cells (150,000 cells/500 µl) were incubated with the various treatments for 30 min. An asterisk indicates significant difference (p<0.05) from control.
Figure 17. Intracellular free calcium concentrations ([Ca$^{2+}$]$_i$) in luteal cells following PGF$_{2\alpha}$ (500 ng/ml) treatment alone or in the presence of hCG (500 ng/ml). The data were standardized to the onset of the peak, which began 25-30 sec after the initiation of treatment. Only data from positively responding luteal cells (n= 28) were depicted. The increase in [Ca$^{2+}$]$_i$ after PGF$_{2\alpha}$ treatment was significant (p<0.01) for positively responding cells. This response was not altered by hCG (p>0.10). The standard error of the means from the model used to analyze the data was 71.3.
Figure 18. Progesterone production by luteal cells incubated for 8 h with increasing doses of PGF$_{2\alpha}$ in the absence and presence of hCG (500 ng/ml). HCG significantly stimulated (p<0.05) progesterone production in the absence of PGF$_{2\alpha}$. This response was significantly inhibited (p<0.05) by PGF$_{2\alpha}$. The standard error of the means from the model used to analyze the data was 0.82.
References


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CHAPTER V
Activation of the Phosphatidylinositol Pathway in the
Primate Corpus Luteum by Prostaglandin E2

Introduction

As in most species, prostaglandin (PG) E2 stimulates progesterone production by the primate corpus luteum (CL) (1-5). These stimulatory effects are associated with activation of the adenylate cyclase system (2, 3, 6, 7). Several studies, however, have reported that the ability of PGE2 to stimulate luteal function in the primate diminishes as the luteal phase progresses (1, 2, 8). In fact, Stouffer et al. (1) reported that PGE2 inhibited gonadotropin-stimulated progesterone production in mid-late luteal phase CL. The mechanisms leading to the loss of the stimulatory effects and/or appearance of the inhibitory effects of PGE2 in the latter stages of the luteal phase have not been elucidated.

Recent evidence suggests that luteolytic responses in the primate CL may be mediated by the phosphatidylinositol second messenger system. Activation of this pathway initiates the hydrolysis of phosphatidylinositol 4,5-bis-phosphate to inositol triphosphate (IP3) and diacylglycerol
(DAG). DAG activates protein kinase C (9) and IP₃ stimulates release of calcium from intracellular stores (10). These events are important in the initiation of a number of physiological responses.

Several lines of evidence support the notion that the phosphatidylinositol pathway may be involved in regression of the primate CL. Calcium inhibits the responsiveness of adenylate cyclase in isolated luteal membranes from the primate (11, 12). In addition, treatment of primate luteal cells with various calcium ionophores inhibits basal and hCG-stimulated progesterone production (13, 14). Thus, mimicking the activity of the phosphatidylinositol pathway elicits inhibitory responses in the primate CL. In addition, a potential luteolysin in the primate, PGF₂α, activates the phosphatidylinositol pathway in monkey luteal cells (15) and human granulosa-luteal cells (16). Activation by PGF₂α is characterized by large increases in phosphatidylinositol hydrolysis (15-17) and a dramatic rise in intracellular free calcium concentrations ([Ca²⁺]ᵢ) (15, 17). In contrast, gonadotropin, which stimulates luteal function, does not increase inositol phosphate accumulation and produces only slight elevations in [Ca²⁺]ᵢ (17). Thus, luteolytic agents are more potent activators of the phosphatidylinositol pathway than luteotropins.

Perhaps, the decline in the stimulatory effects and/or appearance of the inhibitory effects of PGE₂ in the late
luteal phase result from activation of the phosphatidylinositol pathway. Prostaglandins of the E series have been shown to increase inositol phosphate accumulation and $[\text{Ca}^{2+}]_i$ in a number of cell types (18-20), including bovine luteal cells (21-23). The effects of PGE$_2$ on the activity of the phosphatidylinositol pathway have not been examined in the primate CL.

Similar to other species, the primate CL is composed of at least two different types of luteal cells (24, 25) which display differences in size, morphology, and function (8, 24, 25). The responsiveness of the phosphatidylinositol pathway to various physiological agents differs between large and small luteal cells of other species (26, 27). Since functional differences also exist within primate luteal subpopulations (8, 24, 25), important variations in activation of the phosphatidylinositol pathway might exist among these cell types.

The objectives of the current study were to reassess the effects of PGE$_2$ on primate luteal cells collected during the late luteal phase (Expt. 1) and to examine the ability of PGE$_2$ to activate the phosphatidylinositol pathway in the primate CL (Expt. 2). In Expt. 2, we also sought (1) to determine if the responsiveness of the phosphatidylinositol system to PGE$_2$ depends on the stage of the luteal phase of the menstrual cycle, (2) to examine whether this responsiveness is altered during rescue of the CL by in vivo
CG treatments which simulate early pregnancy, and (3) to
determine whether these responses differ with luteal cell
type.

Materials and Methods

Treatments of Animals

The housing and general care of rhesus monkeys was as
described previously (28). Briefly, monkeys were checked
daily for menses, and menstrual records were maintained
(onset of menses = Day 1 of the menstrual cycle). Animals
exhibiting regular cycles of approximately 28 days were used
in the current study. Blood samples were collected daily by
femoral venipuncture at 0800-1000 h from Day 8 of the
menstrual cycle until luteectomy. These samples were used
to determine CL age and confirm normal luteal function. The
serum was stored at -20 C until assayed for hormones. On
Day 14 of the cycle, serum concentrations of 17β-estradiol
were determined by rapid RIA (29). The day of the LH surge
was estimated to occur 1 day before the precipitous decline
in serum estrogen that followed the midcycle estrogen peak
(30).

A midventral laparotomy was performed for CL
collection. Monkeys were anesthetized (28) before surgery
with ketamine hydrochloride (Bristol Laboratories, Syracuse,
NY; 10-15 mg/kg i.m.) supplemented with atropine sulfate
(ESI Pharmaceuticals, Cherry Hill, NJ; 0.02 mg/kg, i.m.) and
sodium pentobarbital (Butler, Columbus, OH; 8-12 mg/kg, i.v.). The CL were excised from the ovaries by blunt dissection and immediately placed in Ham's F-10 medium (Sigma, St. Louis, MO) at 4 C for transport to the laboratory.

For Expt. 1, CL (n=3) were collected during the late luteal phase (d12-13 after the estimated LH surge). For Expt. 2, CL were collected at various times after the estimated LH surge: d 4-5 (early luteal phase; n=4), d 8-9 (mid-luteal phase; n=3), and d 13-14 (late luteal phase; n=4). CL (n=4) were also collected following 1 day of in vivo hCG treatment (15 IU given at 0800 and 1700 h) which began during the mid-luteal phase (d 8-10 after the estimated LH surge). This represents the first day (d 1 of simulated early pregnancy) of a 10-day hCG treatment regimen which invokes pregnancy-like patterns of circulating CG, progesterone, and estradiol in nonpregnant rhesus monkeys (31). CL were collected one day after the initiation of in vivo hCG treatment. During this period of simulated early pregnancy, the corpus luteum is in the initial stages of rescue, as evidenced by increasing levels of circulating progesterone in response to hCG (31).

Preparation and Treatment of Tissue

Dispersed luteal cells were prepared separately from each CL by a combination of gentle mechanical agitation and digestion with 0.25% collagenase (Worthington Biochemical,
Freehold, NJ) and 0.02% deoxyribonuclease (Sigma) (28). Cell viability, as determined by trypan blue exclusion, was 90-95%.

In Expt. 1, suspensions of dispersed luteal cells were incubated for 4 h at 37 C with increasing concentrations of PGE₂ (0, 0.05, 5, 50, 500 ng/ml) in the absence and presence of hCG (100 ng/ml). Following incubation, the cells and medium were stored at -20 C. Prior to radioimmunoassay of the medium, cellular debris was removed by centrifugal separation (160 g).

In Expt. 2, an aliquot of 400,000 cells was removed from the dispersed cell suspension for the measurement of [Ca²⁺]ᵢ by fura-2 fluorescence photometry. The remainder of the luteal cells were washed twice in medium 199 (Sigma). These cells were used to monitor phosphatidylinositol hydrolysis in response to PGE₂ as described below.

**Measurement of Phosphatidylinositol Hydrolysis**

Phosphatidylinositol hydrolysis was monitored by quantifying the amount of labelled inositol phosphates liberated (32) following treatment with PGE₂. Phosphatidylinositol within the cellular membrane was labelled by incubating dispersed cells with 0.5 ml of 3H-myo-inositol (100 μCi/ml; NEN Research Products, Boston, MA; specific activity = 15.4 Ci/mmol) in medium 199 at 37 C for 3h. The cells were then washed twice in fresh medium, incubated for another 15 min, and washed again to remove
unincorporated $^3$H-myo-inositol. Labelled cells were aliquoted into culture tubes at a concentration of 150,000 cells/500 μl. Following a 5 minute pre-incubation with LiCl (10 mM), cells were incubated for 30 min with increasing concentrations of PGE$_2$ (0, 5, 50, 500 ng/ml) in medium 199 with 10 mM LiCl. Lithium was included in the incubation medium to prevent the conversion of inositol phosphates to free inositol. The reaction was terminated by the addition of 170 μl of 10% (w/v) HClO$_4$. Following neutralization with KOH and saturated HEPES solution, tubes were placed in an ice bath for 30 min to precipitate KClO$_4$. The precipitate was pelleted by centrifugation at 2500 x g for 15 min, and the supernatant was stored at -20 C until chromatography.

To isolate inositol phosphates, samples were chromatographed as described by Bone et al. (32) on columns of AG 1-X8 resin (0.6 g; formate form) equilibrated with 10 ml of prep buffer (5 mM Na$_2$B$_4$O$_7$, 0.5 mM EDTA). Samples were thawed and applied to the column in 4 ml of prep buffer. $^3$H-labelled products were eluted by the sequential addition of 10 ml H$_2$O; 20 ml 5 mM Na$_2$B$_4$O$_7$, 60 mM ammonium formate; 20 ml 5 mM Na$_2$B$_4$O$_7$, 150 mM ammonium formate (IP eluted); 20 ml 0.1 m formic acid, 0.4 M ammonium formate (IP$_2$ eluted); and 20 ml 0.1 M formic acid, 1 M ammonium formate (IP$_3$ eluted). The validity of this chromatography procedure was confirmed by following the elution of $^3$H-inositol and $^3$H-IP through the columns. As expected, $^3$H-inositol was eluted by the
first two solutions and $^3$H-IP was eluted by the third solution. For all samples five ml fractions were collected from the columns. Two ml aliquots from these fractions were counted in Ultima Gold counting cocktail (Packard). The counting efficiency was not different among the various buffers. Since endogenous phosphatases convert IP$_3$ to IP$_2$ and IP$_2$ to IP, cpm recovered from these three fractions were combined to quantify the amount of labelled phosphatidylinositol hydrolyzed. The data were presented as fold-change from control to reflect PGE$_2$-induced phosphatidylinositol hydrolysis.

Measurement of [Ca$_{2+}$]$_i$

[Ca$_{2+}$]$_i$ were measured using fura-2 fluorescence photometry of single cells (33). The use of this technique in primate luteal cells has been described previously (14, 15). A Zeiss IM microscope and a 63X PLAN NEOFUAR objective were used. Prior to microscopy, aliquots of 200,000 cells were incubated in 1 ml of F-10 medium with fura-2AM (5 µM) for 30 min at 37 C in a 95% O$_2$:5% CO$_2$ atmosphere. Cells were then washed and incubated for 60 additional min to allow for complete hydrolysis of the fura-2 ester. Luteal cells were then allowed to settle and become loosely attached to a perfusion chamber before perfusion began. The ratio of emission (420-620 nm) of fura-2 at 348/380 nm excitation was measured in small (17-21 µ) and large (23-28 µ) luteal cells (n=4-5 CL/group; 6-12
cells/CL) from 0-3 min. The majority of cells within these two populations are steroidogenic (25). A third population of smaller luteal cells was not studied. The latter population contains the majority (97.4%) of non-steroidogenic cells (25). Cells exhibiting morphological features characteristic of steroidogenic cells, such as a high content of lipid droplets, were selected for study. Treatment with PGE₂ (500 ng/ml) was begun 20-25 sec after initiation of measurement and was continued to 3 min. The ratio of emission at 348/380 nm excitation was converted to [Ca²⁺]ᵢ using the methods of Li et al. (33), as updated by Groden et al. (34).

**RIA**

RIA was used to measure concentrations of progesterone in samples of F-10 medium incubated with dispersed cells, as validated previously (28). Specific and proven antibody for progesterone (Dr. Gordon Niswender, Colorado State University) was donated. Tritiated progesterone was purchased from New England Nuclear (Boston, MA). Samples from individual CL were run within the same assay. The mean coefficient of variation within assays was 11.39%.

**Statistical Analyses**

Analyses were performed using the following statistical programs: Statistical Analysis System (SAS Institute Inc., Cary, NC) and/or Number Cruncher Statistical System (J.L. Hintze, Kaysville, UT).
The effects of PGE$_2$ on basal and gonadotropin-stimulated progesterone production (Expt. 1) were analyzed separately using two-way analyses of variance. The main effects were treatment (PGE$_2$) and CL (block).

The amounts of total labelled inositol phosphates accumulated in response to PGE$_2$ (Expt. 2) were compared using an analysis of variance. The main effects were treatment and reproductive stage with a block on CL. The interaction between treatment and stage was also included in the model.

[Ca$^{2+}$]$_i$ following treatment with PGE$_2$ (Expt. 2) were analyzed using an analysis of variance with treatment, cell type (large or small) and reproductive stage as the main effects and individual cell as a block nested within cell type. The interactions between cell type and treatment, treatment and stage, and cell type and stage were also included in the analysis. The proportion of cells responding to PGE$_2$ with an increase in [Ca$^{2+}$]$_i$ was compared across the different stages using a one-way analysis of variance.

**Results**

**Expt. 1**

PGE$_2$ inhibited basal (p<0.01) and tended to inhibit hCG-stimulated (p=0.07) progesterone production in a dose
dependent manner in CL collected during the late luteal phase (Figure 19).

Expt. 2

Total inositol phosphate accumulation in response to treatment with increasing concentrations of PGE₂ is shown in Figure 20. Overall, PGE₂ stimulated (p<0.01) phosphatidylinositol hydrolysis. This stimulation was not apparent in CL collected during the early luteal phase but was manifested in CL collected later in the luteal phase and during simulated early pregnancy.

Most luteal cells responded to PGE₂ with an unequivocal increase in [Ca²⁺]ᵢ. The remaining cells did not display any change (p>0.10) in [Ca²⁺]ᵢ following PGE₂ treatment. The cells with a positive response to PGE₂ had a dramatic and transient increase in [Ca²⁺]ᵢ (p<0.01, Figure 21). This increase in [Ca²⁺]ᵢ occurred within one minute after the initiation of PGE₂ treatment. The pattern of [Ca²⁺]ᵢ following PGE₂ treatment did not differ with the stage/state from which the CL were collected. Therefore, figure 21 represents data pooled from the various stages/states. However, the proportion of cells responding to PGE₂ with an increase in [Ca²⁺]ᵢ varied significantly with stage/state (Table 5). This proportion was much lower in early luteal phase CL, as compared to CL collected mid or late luteal phase. The pattern of change in [Ca²⁺]ᵢ and the proportion of cells responding with an increase in [Ca²⁺]ᵢ was similar
in small and large luteal cells at all stages/states (data not shown).

Discussion

This study demonstrates that, in addition to its ability to stimulate primate luteal function (1-5), PGE\textsubscript{2} can inhibit progesterone production by luteal cells harvested during the late luteal phase. This is in agreement with the studies of Stouffer et al. (1). Other studies, in which inhibitory responses to PGE\textsubscript{2} were not evident, have shown that the stimulatory effects of PGE\textsubscript{2} observed \textit{in vitro} in the early luteal phase diminish in the mid-late luteal phase (2, 8). Similarly, \textit{in vivo} administration of PGE\textsubscript{2} into the primate CL during the mid-luteal phase does not stimulate circulating progesterone levels at moderate doses (5). In fact, intraluteal infusion of PGE\textsubscript{2} at high doses induces premature luteolysis in the majority of monkeys (5). Thus, PGE\textsubscript{2} may exhibit inhibitory, or at least diminished stimulatory, effects on the primate CL in the latter half of the luteal phase.

This study also indicates that PGE\textsubscript{2} activates the phosphatidylinositol pathway in the primate CL, as evidenced by increases in phosphatidylinositol hydrolysis and \([Ca^{2+}]_{i}\). Increases in inositol phosphate accumulation (21, 22) and \([Ca^{2+}]_{i}\) (23) in response to PGE\textsubscript{2} have also been reported in the bovine CL. Increases in \([Ca^{2+}]_{i}\) induced by calcium
ionophores also inhibit luteal progesterone production in
the primate (13, 14). Thus, the increase in [Ca\(^{2+}\)]\(_{i}\), as
observed in Expt. 2, may mediate the inhibitory effects of
PGE\(_2\) (Expt. 1, ref. 1) on luteal progesterone production in
the late luteal phase.

The notion that the inhibitory effects of PGE\(_2\) are
mediated by the phosphatidylinositol pathway is supported by
the observation that the responsiveness of this pathway to
PGE\(_2\) develops during the latter portion of the luteal phase,
the same period in which inhibitory effects of PGE\(_2\) have
been observed. During the mid-late luteal phase, the
ability of PGE\(_2\) to stimulate inositol phosphate accumulation
becomes apparent. In addition, the proportion of luteal
cells responding to PGE\(_2\) with an increase in [Ca\(^{2+}\)]\(_{i}\) is much
greater in the mid-late luteal phase than in the early
luteal phase. Thus, the ability of PGE\(_2\) to activate the
phosphatidylinositol pathway is associated with the ability
of PGE\(_2\) to inhibit luteal function in the primate.

Previous studies from our laboratory have shown that
the luteolytic effects of another prostaglandin, PGF\(_{2\alpha}\), are
also associated with activation of the phosphatidylinositol
pathway (15). Interestingly, the patterns of response to
PGE\(_2\), in terms of phosphatidylinositol hydrolysis and
[Ca\(^{2+}\)]\(_{i}\), and the changes in responsiveness of this pathway
to PGE\(_2\) over the luteal phase are very similar to that of
PGF\(_{2\alpha}\) (15). As observed with PGE\(_2\), the responsiveness of
the phosphatidylinositol pathway to PGF$_{2\alpha}$ is low during the early luteal phase and increases by late luteal phase (15). However, the magnitude of the response to PGE$_2$ (current study), in terms of inositol phosphate accumulation and [Ca$^{2+}$]$_i$, is about 50% smaller than that of PGF$_{2\alpha}$ (15). Studies in the bovine CL have also reported that PGF$_{2\alpha}$ is more potent than PGE$_2$ in activating the phosphatidylinositol pathway (21-23).

Another notable difference between the response of the phosphatidylinositol pathway to PGE$_2$ and PGF$_{2\alpha}$ is apparent in the simulated early pregnancy group. Whereas the \textit{in vivo} hCG treatment of simulated early pregnancy enhances the responsiveness of the phosphatidylinositol pathway to PGF$_{2\alpha}$ (15), the responsiveness to PGE$_2$ is not augmented by this treatment. The significance of this difference is unclear. Nonetheless, the similarities in the activation of the phosphatidylinositol pathway by PGE$_2$ and PGF$_{2\alpha}$ are striking. These observations, coupled with the fact that both prostaglandins inhibit progesterone production in the late luteal phase, suggests that primate luteal regression may result from activation of this pathway by both PGF$_{2\alpha}$ and PGE$_2$.

Other research groups have suggested that PGE$_2$-induced activation of the phosphatidylinositol pathway in the bovine CL results from cross-reactivity of PGE$_2$ with the PGF$_{2\alpha}$ receptor (21). This notion is based on the fact that a much
higher concentration of PGE\textsubscript{2}, as compared to PGF\textsubscript{2α}, is required to increase inositol phosphate accumulation in the bovine CL. In fact, the PGE\textsubscript{2} concentration is comparable to that required to significantly cross-react with the PGF\textsubscript{2α} receptor (35). In the primate CL, about 100 times more PGE\textsubscript{2} than PGF\textsubscript{2α} is required to displace 50% of labelled PGF\textsubscript{2α} from its receptor (36). The current study demonstrated that the response to PGE\textsubscript{2}, in terms of phosphatidylinositol hydrolysis, appears dose-dependent in the range examined. This response to PGF\textsubscript{2α} has also been shown to be dose-dependent in the same range (15). In subsequent examinations, we have also observed that increases in [Ca\textsuperscript{2+}]	extsubscript{i} can be induced by lower treatment concentrations (50 ng/ml) of PGE\textsubscript{2}, as well as PGF\textsubscript{2α} (unpublished observations). Since the responses to PGF\textsubscript{2α} and PGE\textsubscript{2} exhibit similar dose-dependency in the same range of concentrations, it is unlikely that the activation of the phosphatidylinositol pathway by PGE\textsubscript{2} in the primate CL results from cross-reactivity of PGE\textsubscript{2} with the PGF\textsubscript{2α} receptor.

In conclusion, this study demonstrates that PGE\textsubscript{2} activates the phosphatidylinositol pathway in the primate CL. This activation is augmented as the luteal phase progresses. Thus, the inhibitory effects of PGE\textsubscript{2} on luteal progesterone production observed in the late luteal phase
are associated with activation of the phosphatidylinositol pathway.
Figure 19. Progesterone production by luteal cells (n=3 CL) collected during the late luteal phase. Luteal cells (12,500 cells/250 µl) were incubated for 4 h with increasing concentrations of prostaglandin (PG) E$_2$ in the absence and presence of human chorionic gonadotropin (hCG). PGE$_2$ inhibited basal (p<0.05) and hCG-stimulated (p=0.07) progesterone production. The standard error of the means from the models used to analyze the data were 0.19 in the absence of hCG and 0.48 in the presence of hCG.
Figure 20. Fold change in total inositol phosphate (IPs) accumulation after PGE$_2$ treatment in primate luteal cells collected from early (n=4 CL), mid (n=3 CL), and late (n=4 CL) luteal phase (LP) and following 1 day of in vivo hCG treatment (d 1 of simulated early pregnancy (SEP d1); n=4 CL). Luteal cells (150,000 cells/500 µl) were incubated in the absence and presence of increasing doses of PGE$_2$ for 30 min. The main effect of PGE$_2$ was significant (p<0.05).
Figure 21. Intracellular free calcium concentrations ([Ca$^{2+}$]$_i$) after PGE$_2$ treatment in primate luteal cells (n=15 CL). The data were pooled across stage/state of the luteal phase. The data were standardized to the onset of the peak, which began within one minute after the initiation of PGE$_2$ treatment. Only data from positively responding luteal cells (n=46) were depicted. The increase in [Ca$^{2+}$]$_i$ after PGE$_2$ was significant (p<0.01) for positively responding cells.
Table 5. Proportion (mean±SE) of Luteal Cells (n=15 CL, 6-12 cells/CL) Responding to PGE$_2$ with an Increase in Intracellular Free Calcium at Various Stages/States of the Luteal Phase.

<table>
<thead>
<tr>
<th>Stage/State</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Luteal Phase</td>
<td>0.12±.06$^a$</td>
</tr>
<tr>
<td>Mid-Luteal Phase</td>
<td>0.63±.07$^b$</td>
</tr>
<tr>
<td>Late Luteal Phase</td>
<td>0.66±.13$^b$</td>
</tr>
<tr>
<td>Simulated Early Pregnancy (d 1)</td>
<td>0.45±.18$^{ab}$</td>
</tr>
</tbody>
</table>

Means with different letter designations displayed significant differences in proportion (p<0.05).
References


CHAPTER VI
Modulation of Primate Luteal Function by Various Activators of Protein Kinase C

Introduction
Several lines of evidence suggest that the Ca$^{2+}$-activated, phospholipid-dependent kinase described by Nishizuka (1) plays a role in the regulation of the corpus luteum (CL). Activity of this kinase, termed protein kinase C, has been demonstrated in the CL of various species (2-7). In addition, experimental activation of protein kinase C with phorbol esters modulates luteal progesterone production in these species (7-14). Activation of protein kinase C in response to physiological agents, such as PGF$_2\alpha$, has also been demonstrated in the CL (5, 15).

Protein kinase C activation comprises one component of the phosphatidylinositol signalling system. Activation of the phosphatidylinositol system initiates the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol triphosphate (IP$_3$) and diacylglycerol (DAG). DAG activates protein kinase C (1) and IP$_3$ stimulates release of calcium from intracellular stores (16).

Several lines of evidence support the notion that the
phosphatidylinositol pathway may be involved in regression of the primate CL. Mimicking the activity of the phosphatidylinositol pathway with calcium ionophores elicits inhibitory responses in the primate CL (17, 18). In addition, agents which exhibit luteolytic effects are potent activators of the phosphatidylinositol pathway in primate luteal (19, 20) and granulosal-luteal cells (21). Activation of the phosphatidylinositol pathway in the above studies was characterized by changes in the accumulation of inositol phosphates and changes in intracellular free calcium concentrations ([Ca^{2+}]_i). Few studies have directly examined the potential role of the protein kinase C component of this pathway in the regulation of the primate CL.

Protein kinase C activity has been reported in the primate ovary (22). In addition, studies with human granulosal-luteal cells have demonstrated inhibitory effects of phorbol ester on gonadotropin-stimulated cAMP (21) and progesterone (23) production. Further studies are necessary to understand the role of protein kinase C in the regulation of the primate CL.

The objective of the current study was to characterize the response of primate luteal cells to two activators of protein kinase C, phorbol ester and synthetic diacylglycerol.
Materials and Methods

Treatments of Animals

The housing and general care of rhesus monkeys at The Ohio State University was described previously (24). Monkeys were checked daily for menses, and menstrual records were maintained (onset of menses = Day 1 of the menstrual cycle). Animals exhibiting regular cycles of approximately 28 days were used in the current study. Blood samples were collected daily by femoral venipuncture at 0800-1000 h from Day 8 of the menstrual cycle until luteectomy. The serum was stored at -20 C until assayed for hormones. On Day 14 of the cycle, serum concentrations of 17β-estradiol were determined by rapid RIA (25). The day of the LH surge was estimated to occur 1 day before the precipitous decline in serum estrogen that followed the midcycle estrogen peak (26).

CL (n=10) were collected via midventral laparotomy 8-9 days after the estimated day of the LH surge. Monkeys were anesthetized (24) before surgery with ketamine hydrochloride (Bristol Laboratories, Syracuse, NY; 10-15 mg/kg i.m.) supplemented with atropine sulfate (ESI Pharmaceuticals, Cherry Hill, NJ; 0.02 mg/kg, i.m.) and sodium pentobarbital (Butler, Columbus, OH; 8-12 mg/kg, i.v.). The CL were excised from the ovaries by blunt dissection and immediately placed in Ham's F-10 medium (Sigma, St. Louis, MO) at 4 C for transport to the laboratory.
Preparation and Treatment of Tissue

Dispersed luteal cells were prepared separately from each CL by a combination of gentle mechanical agitation and digestion with 0.25% collagenase (Worthington Biochemical, Freehold, NJ) and 0.02% deoxyribonuclease (Sigma) (24). Cell viability, as determined by trypan blue exclusion, was >95%. The luteal cells were incubated in duplicate in a gyratory shaker bath at concentrations of 50,000 cells per 250 μl Ham's F-10 medium at 37°C in an atmosphere of 95% O₂:5% CO₂.

To assess the effects of phorbol ester on unstimulated luteal P production, cells were incubated for 1, 2, 4, and 8 h with increasing concentrations of TPA (12-O-tetradecanoylphorbol 13-acetate; 0, 0.5, 5, 50 ng/ml, n=4 CL). This experiment was repeated in the presence of 500 ng/ml hCG (CR 127, 14,900 IU/mg, NIADDK, n=3 CL) to examine the effect on gonadotropin-stimulated progesterone production. The effects of a synthetic diacylglycerol (1,2-dioctanoyl-rac-glycerol, 100 μg/ml) on basal and gonadotropin-stimulated progesterone production were examined in the same manner (n=6 CL).

Cells and medium were frozen at -20°C after incubation. Cells and medium were separated by centrifugation at 160 x g prior to quantification of progesterone concentrations by RIA.
RIA

RIA was used to measure concentrations of progesterone in samples of F-10 medium incubated with dispersed cells, as validated previously (24). Specific and proven antibody for progesterone (Dr. Gordon Niswender, Colorado State University) was donated. Tritiated progesterone was purchased from New England Nuclear (Boston, MA). The mean coefficients of variation within assays and between assays were 15.1% and 16.8%, respectively.

Statistical Analyses

Analyses were performed using the following statistical programs: Statistical Analysis System (SAS Institute Inc., Cary, NC) and/or Number Cruncher Statistical System (J.L. Hintze, Kaysville, UT).

The response to each protein kinase C activator (TPA and DAG) over time was analyzed separately for those luteal cells treated with and without hCG. Three-way analyses of variance with individual CL as a block and TPA (or DAG) and time as the main effects were used to compare progesterone concentrations in these time trials. An interaction between TPA (or DAG) and time was also used in the model.

Results

The effect of TPA on basal progesterone production is depicted in Figure 22. In the absence and presence of TPA, basal progesterone production significantly increased
(p<0.01) over time. TPA significantly (p=0.05) enhanced the basal production of progesterone. The responses to 0.5 and 5 ng/ml of TPA (data not shown) were intermediate between 0 and 50 ng/ml.

As observed with basal production, gonadotropin-stimulated progesterone production significantly increased (p<0.01, Figure 23) over the incubation period. However, TPA tended to inhibit (p=.11) gonadotropin-stimulated progesterone production. The responses to 0.5 and 5 ng/ml of TPA (data not shown) were intermediate between 0 and 50 ng/ml. In a direct comparison of Figure 22 and 23, the stimulation of progesterone production by hCG is not readily apparent. However, different CL were used for the data in each figure. HCG significantly (p<0.05) stimulated progesterone production in the CL used to assess the effects of TPA on gonadotropin-stimulated progesterone production.

In contrast to the effect of TPA, DAG significantly inhibited (p<0.05) the basal production of progesterone (Figure 24). Although not significant (p=.14), hCG-stimulated progesterone production (Figure 25) was also slightly suppressed by DAG in the majority of the CL (5 of the 6 CL examined) at 8 h. This concentration of DAG (100 μg/ml) did not alter the viability of luteal cells (n= 1 CL), which was assessed by trypan blue exclusion at 2 and 8 h.
Discussion

This study demonstrates disparate effects of two putative activators of protein kinase C on progesterone production by primate luteal cells. DAG inhibits, while TPA stimulates, basal progesterone production. However, DAG and TPA both tend to inhibit gonadotropin-stimulated progesterone production. The reason for the disparate responses to DAG and TPA under basal conditions is not clear. Interestingly, in other cell types the temporal pattern of stimulation of protein kinase C by TPA and DAG differs (27). Activation of protein kinase C by TPA is rapid yet transient. In contrast, the increase in protein kinase C activity after DAG treatment is more sustained. Furthermore, down-regulation of protein kinase C is evident in many cell types after TPA (27, 28) but not DAG (27) treatment. In fact, down-regulation of protein kinase C by TPA has been observed in the ovine CL (7, 15). Progesterone production in these protein kinase C-deficient luteal cells tends to be enhanced (7). Perhaps the stimulatory effects of TPA observed in the current study reflect down-regulation of protein kinase C. In addition to an ability to down-regulate protein kinase C, TPA has been shown in some studies to act independently of protein kinase C (29, 30). Therefore, the responses to DAG may more accurately reflect the steroidogenic response of primate luteal cells to an increase in protein kinase C activity.
The effects of TPA on luteal steroidogenesis in other species vary from stimulatory (8, 11) to inhibitory (7, 9, 12, 13). This variation may result from differences among species or the use of various experimental conditions, leading to either activation of protein kinase C by TPA or the down-regulatory and nonspecific effects of TPA. Unfortunately, the effects of TPA on luteal function in these nonprimate species have not been compared to those of DAG.

In the current study, luteal progesterone production by the primate CL was inhibited by DAG. The notion that these inhibitory responses in the primate CL result from increased protein kinase C activity is substantiated by studies in human granulosa-luteal cells (21, 23). Treatment of human granulosa-luteal cells with an activator of protein kinase C inhibits gonadotropin-stimulated cAMP (21) and progesterone (23) production. Although TPA was used to activate PKC in these studies, the use of short treatment periods (30 min) may have avoided the down-regulatory and nonspecific effects of TPA. Since activation of protein kinase C (current study) and increases in \([Ca^{2+}]_i\) (17, 18) appear to inhibit luteal progesterone production, both of these components of the phosphatidylinositol pathway may be involved in the inhibitory responses to agents shown to activate this pathway in the primate CL (PGF \(_2\alpha\) [19] and PGE \(_2\) [20]).
Although we have examined the luteal effects of each component of the phosphatidylinositol pathway (protein kinase C and intracellular free Ca\(^{2+}\)) separately, these two interacting components would be activated simultaneously in most physiological circumstances. Further studies in the primate CL are necessary to determine whether the responses to protein kinase C activators are enhanced, or otherwise altered, by changes in \([Ca^{2+}]_i\). Such interactions have been observed in the bovine CL (31).

In summary, this study provides preliminary evidence for the hypothesis that enhanced protein kinase C activity plays a role in primate luteal regression. This study also emphasizes the need for careful selection of protein kinase C activators and consideration of the experimental conditions under which these agents are used.
Figure 22. Progesterone concentrations in medium incubated for various times with luteal cells (50,000 cells/250 µl; n=4 CL) in the absence and presence of a protein kinase C-activating phorbol ester (TPA, 50 ng/ml). Treatment with TPA stimulated (p=0.05) the time-dependent increase in basal progesterone production.
Figure 23. Progesterone concentrations in medium incubated for various times with luteal cells (50,000 cells/250 μl; n=4 CL) treated with 500 ng/ml hCG (human chorionic gonadotropin) in the absence and presence of 50 ng/ml TPA. Treatment with TPA tended to inhibit (p=0.11) gonadotropin-stimulated progesterone production.
Figure 24. Progesterone concentrations in medium incubated for various times with luteal cells (50,000 cells/250 μl; n=6 CL) in the absence and presence of a synthetic diacylglycerol (DAG, 100 μg/ml). Treatment with DAG attenuated (p<0.05) the time-dependent increase in basal progesterone production.
Figure 25. Progesterone concentrations in medium incubated for various times with luteal cells (50,000 cells/250 µl; n=6 CL) treated with 500 ng/ml hCG in the absence and presence of 100 µg/ml DAG.
References


CONCLUSIONS

From the studies described and reviewed in this dissertation, a hypothesis for the role of prostaglandins and the phosphatidylinositol pathway in the regulation of the primate CL has been formulated. During the early part of the luteal phase, luteotropic PGs, such as PGE$_2$ and PGI$_2$, may enhance the LH-induced development and support of the CL. This seems likely since luteal production of these PGs is high during the early luteal phase, the same period when these PGs stimulate luteal progesterone production in vitro (1-7). In addition, blockade of luteal prostaglandin production during the midluteal phase results in premature regression of the CL (8). Thus, luteotropic actions of some PGs may be necessary for normal luteal function. The luteotropic effects of these PGs, as well as LH, appear to be mediated by the adenylate cyclase pathway (3, 4, 9-11).

In contrast, luteolysis may be mediated by the phosphatidylinositol pathway in the primate CL. Calcium ionophores, which increase intracellular free calcium concentrations ([Ca$^{2+}$]$_i$), and synthetic diacylglycerol, which activates protein kinase C, inhibit luteal progesterone production. In addition, calcium ionophores
stimulate luteal PG production. Thus, artificial induction of the phosphatidylinositol pathway in midluteal phase CL elicits patterns of luteal progesterone and prostaglandin production resembling that found in untreated luteal cells from the late luteal phase. The observation that a luteolytic agent (PGF$_{2\alpha}$) is a more potent activator of the phosphatidylinositol pathway than a luteotropin (hCG) lends additional support to the notion that this pathway mediates luteolysis. Furthermore, activation of the phosphatidylinositol pathway by both PGF$_{2\alpha}$ and PGE$_2$ is greatest in the latter part of the luteal phase, when these agents exhibit luteolytic effects.

These studies also suggest that a combination of changes in luteal PG production and luteal responsiveness to these PGs plays a role in regulating luteal lifespan. Production of all PGs are high during the early luteal phase. As previously mentioned, it is at this time that PGE$_2$ and PGI$_2$ are potent stimulators of luteal function. PGF$_{2\alpha}$, usually considered a luteolytic PG, shows either no effect (3, 10, 12) or a stimulatory effect (2) on luteal cAMP and progesterone production during the early luteal phase. Therefore, the predominant luteotropic influence of these PGs in the early luteal phase may facilitate luteal development and support.

In the midluteal phase, the luteolytic effects of PGF$_{2\alpha}$ first appear (2, 3, 10, 12). Some studies have also
shown that the luteotropic effects of PGE$_2$ are diminishing at this time (2, 3, 13). The ability of PGF$_{2\alpha}$ and PGE$_2$ to activate the phosphatidylinositol pathway also increases in the latter part of the luteal phase. Although luteal tissue from the midluteal phase is responsive to the luteolytic actions of PGF$_{2\alpha}$, and possibly PGE$_2$, luteal production of these PGs is low during the midluteal phase. This suppressed PG production during the midluteal phase may prevent premature regression of the primate CL.

By late luteal phase, luteal PGF$_{2\alpha}$ production is again elevated. PGE$_2$ production also increases from mid- to late luteal phase. This elevated PG production coupled with enhanced ability of these PGs to activate the phosphatidylinositol pathway and suppress progesterone production may lead to the initiation of luteolysis during the late luteal phase.

Since activation of the phosphatidylinositol pathway by these PGs results in increased [Ca$^{2+}$]$_i$ and increases in [Ca$^{2+}$]$_i$ result in enhanced luteal PG production, it is possible that luteolysis in the primate results from a positive feedback loop. The question that then arises is how this positive feedback loop is initiated. A similar dilemma exists in the theory of luteolysis in the domestic species. The positive feedback loop initiating luteolysis in the domestic species is thought to involve luteal oxytocin and uterine prostaglandins. PGF$_{2\alpha}$ stimulates
luteal oxytocin production and oxytocin stimulates uterine PG production (see 14 for review). It has been proposed that the initial event in this positive feedback loop is elevated uterine PGF$_{2\alpha}$ production (15). However, the mechanisms which result in this initial enhancement of uterine PGF$_{2\alpha}$ production are not clear. McCracken et al. (16) have suggested that neurohypophyseal oxytocin is the trigger to initiate pulsatile PGF$_{2\alpha}$ secretion from the uterus. In addition, a certain period of uterine exposure to both estrogen and progesterone appears necessary for the induction of luteolytic pulses of PGF$_{2\alpha}$ (see 14 for review). Perhaps luteal PG production in the primate is regulated in a similar manner by estrogen and progesterone. Since oxytocin is also a potential luteolytic agent in the primate, it may trigger the positive feedback loop proposed to initiate regression of the primate CL. In fact, oxytocin increases uterine PG production in the domestic species by activating the phosphatidylinositol pathway (17). The role of oxytocin, however, in primate luteal regression remains speculative. Thus, though it appears plausible that luteolysis in the primate involves luteal PGs and activation of the phosphatidylinositol pathway, many questions about this proposed mechanism of luteolysis remain unanswered.

In fact, although the hypothesis that PGs and the phosphatidylinositol pathway are involved in primate luteal regression is strongly supported by the studies described
within this dissertation, conclusive evidence supporting the hypothesis is still lacking. It is essential to determine whether blocking the action of PGF$_{2\alpha}$ and/or PGE$_2$ on the CL prevents luteal regression, whether the in vitro and in vivo luteolytic effects of these agents are prevented by blockade of the phosphatidylinositol pathway, and whether luteal regression is prevented by interruption of the phosphatidylinositol pathway. Some of these experiments have been performed in other species in which PGF$_{2\alpha}$-induced activation of the phosphatidylinositol pathway is proposed to be a mediator of luteolysis. Behrman and his colleagues found that preventing PGF$_{2\alpha}$-induced increases in [Ca\(^{2+}\)]$_i$ did not prevent the suppression of in vitro cAMP production by PGF$_{2\alpha}$ in the rat CL (18). Similarly, preventing protein kinase C activation did not abrogate the effects of PGF$_{2\alpha}$ on luteal cAMP production in this species (19). Although these studies indicate that suppression of cAMP production by PGF$_{2\alpha}$ is not mediated by the phosphatidylinositol pathway in the rat CL, the possibility that this pathway is involved in more distal actions of PGF$_{2\alpha}$ on steroidogenesis has not been tested. In the sheep CL, depletion of protein kinase C does prevent the inhibitory effects of PGF$_{2\alpha}$ on progesterone production (20). Perhaps, activation of the phosphatidylinositol pathway is also necessary for the action of PGF$_{2\alpha}$ on the primate luteal cell. It has been shown in human granulosal-luteal cells that down-regulation
of protein kinase C prevents PGF$_{2\alpha}$ from stimulating the activity of phosphodiesterase (21), an enzyme that catalyzes cAMP metabolism. This suggests that protein kinase C may mediate the inhibitory effects of PGF$_{2\alpha}$ on the concentrations of cAMP in human granulosal-luteal cells.

It is very likely that substances other than PGF$_{2\alpha}$ and PGE$_2$ activate the phosphatidylinositol pathway in the primate CL. Possibilities include agents which have been shown to induce phosphatidylinositol hydrolysis and/or increases in $[Ca^{2+}]_i$ in reproductive tissues from other species: oxytocin (17), GnRH (22-24), arachidonic acid (25), and other products of arachidonic acid metabolism (25). Preliminary studies from our laboratory indicate that arachidonic acid, but not GnRH, increases $[Ca^{2+}]_i$ in primate luteal cells. However, the role of these agents in primate luteal regulation has yet to be thoroughly assessed.

If luteolysis in the primate involves activation of the phosphatidylinositol pathway by luteal prostaglandins, it is important to understand how CG overcomes this luteolytic mechanism to rescue the CL during early pregnancy. It is somewhat surprising that PG-induced activity of the phosphatidylinositol pathway is unaffected \textit{(in vitro} hCG) or even enhanced \textit{(in vivo} hCG) by hCG. However, considering that hCG also does not prevent the \textit{in vitro} (2, 10, 12, current studies) or \textit{in vivo} (26, 27) inhibition of luteal progesterone and/or cAMP production by PGF$_{2\alpha}$, maternal
recognition of pregnancy in the primate may not involve direct inhibition of the action of the luteolysin(s). Rather, chorionic gonadotropin may prevent the production of the luteolytic substance(s). In contrast to the high levels of luteal PGF$_{2\alpha}$ production during the late luteal phase of the menstrual cycle, there is no late luteal phase increase in luteal PGF$_{2\alpha}$ production during rescue of the CL by hCG in simulated early pregnancy (28). Similarly, CL treated with hCG in vivo release lower amounts of PGF$_{2\alpha}$ in vitro than age-matched CL in the late luteal phase (29). Thus, although gonadotropin does not prevent exogenous PGF$_{2\alpha}$ from activating the phosphatidylinositol pathway, suppressed production of endogenous PGF$_{2\alpha}$ during early pregnancy may allow for rescue of the CL. Again, the question of regulation of luteal prostaglandin production arises. It is not clear how CG may prevent the late luteal phase rise in luteal prostaglandin production.

Several novel hypotheses regarding luteal regression have emerged in the last decade. It has been proposed that interactions between the steroidogenic luteal cells and other cells found in the CL, particularly immune cells, may be important in luteal regulation (30-34). Nonsteroidogenic cells from the vascular (35, 36), immune (37, 38) and nervous systems (39) are found within the CL. In addition, many studies have demonstrated alterations in the function of the steroidogenic luteal cells when these
nonsteroidogenic cells or their secretions are present (32 <review>, 40-47). Although most of these studies have been performed in nonprimate species, similar mechanisms might be involved in primate luteal regulation (42, 44, 45, 48, 49). Acceptance of these hypotheses may serve to strengthen the hypothesis that luteal prostaglandins and activation of the phosphatidylinositol pathway are involved in luteal regression. It has been known for some time that these nonsteroidogenic cells both produce and respond to PGs (50-53). Therefore, prostaglandins produced by either the nonsteroidogenic cells or the steroidogenic luteal cells may regulate the function of both cell types. In addition to PGs, other secretory products of the cells of the immune (54), nervous (55, 56), and vascular (57) system activate the phosphatidylinositol pathway in their target cells. Thus, perhaps prostaglandins and the phosphatidylinositol pathway are mediators of the interactions between steroidogenic luteal cells and nonsteroidogenic cells found within the CL.

Another theory of luteolysis that has recently emerged is apoptosis. As originally described by Kerr et al. (58), apoptosis is cell death characterized by shrinkage of the cell and condensation and fragmentation of cellular components within the cytoplasm and nucleus. It is possible that luteolysis is an apoptotic process. Both steroidogenic and endothelial cells of the ovine CL show morphological
changes characteristic of apoptosis following a luteolytic dose of PGF$_{2\alpha}$ (59). Interestingly, the early histological studies of the primate CL noted morphological characteristics during luteal regression that are consistent with apoptosis (60). Intracellular free calcium appears to be a key regulator of apoptosis (61). Perhaps, the increases in [Ca$^{2+}$]$_i$ induced by luteal PGs lead to luteolysis in the primate by inducing apoptosis. The presence of a calcium-sensitive endonuclease, known to produce an apoptotic pattern of DNA fragmentation (62), has been demonstrated in the CL (63). Therefore, it is plausible that increases in [Ca$^{2+}$]$_i$ following activation of the phosphatidylinositol pathway may induce luteolysis via apoptosis.

The hypothesis that luteolysis results from the production and action of oxygen radicals and other reactive oxygen species has been proposed recently by Behrman and his colleagues (64). These highly reactive oxygen radicals, which include superoxide anion and hydroxyl radical, possess at least one unpaired electron. Other reactive oxygen species, such as hydrogen peroxide, can be easily converted to oxygen radicals. These reactive oxygen species can result in cell injury or death via damage to DNA and RNA, alterations in cellular membranes by lipid peroxidation, and damage to proteins (see 64 for review). Interestingly, PGF$_{2\alpha}$ increases hydrogen peroxide generation in the CL (65).
and reactive oxygen species have been shown to elevate [Ca\textsuperscript{2+}]\textsubscript{i} in other cell types (66, 67). It is possible that one or more reactive oxygen species mediate the effects of PGF\textsubscript{2α} on the phosphatidylinositol pathway to result in regression of the primate CL.

The preceding review of a few recent hypotheses for luteolysis described in other species reveals complementarity between these theories and the hypothesis for primate luteolysis described in this dissertation. Of course, a great deal of experimentation is still required to substantiate these theories and their link to prostaaglandins and/or the phosphatidylinositol pathway in the CL.

Since the CL is derived from the follicle and the luteal cells retain characteristics similar to the thecal and granulosal cells of the follicle, examining the role of prostaaglandins and the phosphatidylinositol pathway in follicular regulation may provide insights into luteal regulation. The cells of the follicle produce (68 <review>, 69) and respond to prostaaglandins (68 <review>, 70). In fact, an essential role for follicular prostaaglandins in the process of ovulation has, until recently (71, 72), been widely accepted (68). Perhaps, the high levels of PG production in the early luteal phase reflect the high capacity of the mature cells of the follicle to produce these PGs. In addition to the ability to produce and respond to PGs, the follicle displays an active
phosphatidylinositol signalling pathway (73, 74). In many species, agents which activate the phosphatidylinositol pathway in the CL also activate this system in follicular cells (23, 75, 76). Thus, it appears that the cellular mechanisms hypothesized to be involved in luteolysis are functional in the ovary even before development of the CL. However, significant changes in the activity of the phosphatidylinositol pathway and the production and response to PGs occurs during the follicular to luteal phase transition and throughout the luteal phase. It is not clear why the phosphatidylinositol pathway would be responsive during the late follicular, but not early luteal, phase. Perhaps the high PG levels in the late follicular phase lead to down-regulation of PG receptors and/or desensitization of the phosphatidylinositol pathway to PGs in the early luteal phase. The studies described within this dissertation emphasize the need to consider the age of the CL when assessing regulatory mechanisms. Care must also be taken when comparing the apparently similar regulatory mechanisms of the follicle and CL.

In summary, the CL of the primate may be regulated by luteal prostaglandins and the phosphatidylinositol pathway. The response of the CL to these proposed agents and mechanisms of luteolysis is dynamic, exhibiting many changes throughout the luteal phase. Of particular interest for future study are the phenomena which lead to these dynamic
changes and the cellular mechanism(s) by which these luteolytic processes are abrogated during early pregnancy. An understanding of the precise cellular responses (eg. alterations in steroidogenic enzymes, free radical production, apoptosis) following initiation of these luteolytic mechanisms also awaits further study. Similarly, understanding if and how these potential pathways of luteolysis influence and are influenced by cells of the immune, vascular and nervous system may significantly advance our knowledge of the CL and other endocrine systems. The importance of a greater understanding of the primate corpus luteum is immense in these times of world overpopulation and increased infertility. Hopefully, advances in this area will provide a basis for improved fertility control in the woman.

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