Progesterone Inhibits Oxytocin- and Prostaglandin F<sub>2alpha</sub>-Stimulated Increases in Intracellular Calcium Concentrations in Small and Large Ovine Luteal Cells<sup>1</sup>

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ABSTRACT

There is increasing evidence that the corpus luteum has an important role in regulating its own demise. A series of experiments was performed to study the effects of luteal concentrations of progesterone on the functions of steroidogenic luteal cells. In the first experiment, steroidogenic small luteal cells (SLCs) were separated from endothelial cells, and it was determined that it was the SLCs that contained receptors for oxytocin. Treatment with progesterone (95 μM) for as little as 1 h decreased (P < 0.05) the percentage of SLCs responding to oxytocin (10 μM) with an increase in intracellular concentrations of calcium, and this effect continued for the duration of the experiment. In a second experiment, the response to oxytocin was increased (P < 0.05) by 3 h (but not 1 h) following progesterone removal, with a further increase by 16 h. The ability of 1 μM prostaglandin F<sub>2alpha</sub> (PGF<sub>2alpha</sub>) to increase intracellular concentrations of calcium was also decreased (P < 0.05) by progesterone treatment. By 3 h following removal of progesterone, the percentage of steroidogenic large luteal cells (LLCs) responding to PGF<sub>2alpha</sub> was increased and not different from that observed in cells 16 h after progesterone removal. Finally, cyclodextrins (methyl-beta cyclodextrin [MbetaCD]) were used to remove cholesterol from the plasma membrane of luteal cells, and MbetaCD loaded with cholesterol was used to put cholesterol back into the plasma membrane of progesterone-treated cells. Treatment with MbetaCD reduced (P < 0.05) the responsiveness of SLCs to oxytocin and LLCs to PGF<sub>2alpha</sub>. Use of cholesterol-loaded MbetaCD returned the responsiveness of both SLCs and LLCs treated with progesterone to that observed in vehicle (no progesterone)-treated controls. In summary, intraluteal concentrations of progesterone inhibit the ability of oxytocin to increase intracellular concentrations of calcium in SLCs and the ability of PGF<sub>2alpha</sub> to increase intracellular concentrations of calcium in LLCs. The highest concentration of progesterone appears to act by influencing cholesterol content of the luteal cell membrane.

corpus luteum, oxytocin, progesterone, prostaglandin F<sub>2alpha</sub>

INTRODUCTION

The generally accepted model for luteolysis in ruminants is that oxytocin released from the posterior pituitary stimulates the release of prostaglandin F<sub>2alpha</sub> (PGF<sub>2alpha</sub>) from the uterus [1, 2]. Prostaglandin F<sub>2alpha</sub> then stimulates release of oxytocin from large luteal cells (LLCs) in the corpus luteum (CL), which causes more PGF<sub>2alpha</sub> to be released by the uterus in a positive feedback mechanism resulting in a decline in circulating progesterone concentrations and regression of the CL.

There has been increasing experimental evidence that progesterone may be involved in protection of the CL from luteolysis. It has generally been accepted that apoptosis of cells is involved in luteolysis, and it has been suggested that progesterone has antiapoptotic actions in luteal cells. Progesterone suppresses apoptosis in bovine luteal cells by inhibiting Fas and caspase-3 activation [3], which are part of the apoptotic pathway. Moreover, administration of progesterone to rats reduces the number of apoptotic cells during regression of the postpartum CL in the absence of the classical nuclear progesterone receptor [4]. Progesterone also inhibits the ability of bovine luteal cells to elicit T-cell proliferation in vitro, which likely has a role in luteolysis [5].

Progesterone also appears to inhibit binding of oxytocin to its receptor, reducing oxytocin-stimulated secretion of PGF<sub>2alpha</sub> from ovine endometrium [6]. This study was conducted using concentrations of progesterone (16 nM) considered to be physiological for effects on the uterus. Progesterone concentrations >10 μM have been shown to have nonspecific steroid effects on the plasma membrane [7] by disrupting the lipid domains of the membrane [8]. Concentrations of progesterone that disrupt the lipid bilayer of the plasma membrane and cause nonspecific steroid effects are similar to intraluteal concentrations to which steroidogenic luteal cells would be exposed.

Previously, we have shown that 95 μM progesterone decreases the percentage of ovine small luteal cells (SLCs) responsive to oxytocin; however, neither 3 nor 31 μM progesterone was able to cause the same suppression [9]. It was unclear why 3 or 31 μM progesterone did not inhibit oxytocin-stimulated increase in concentrations of intracellular calcium, but we speculated that these concentrations of progesterone were lower than intraluteal concentrations in vivo. Furthermore, the effect of progesterone was steroid specific because 17β-estradiol, testosterone, or cortisol (95 μM) did not decrease the percentage of cells responsive to oxytocin [9]. In the previous study, it was not determined if the cells that responded to oxytocin were steroidogenic cells or endothelial cells. Therefore, the first objective of the present studies was to determine if the cells responsive to the oxytocin-stimulated increase in intracellular calcium were steroidogenic SLCs or endothelial cells. The second objective was to determine if progesterone inhibited the ability of PGF<sub>2alpha</sub> to increase concentrations of intracellular calcium in ovine LLCs. The third objective was to determine how rapidly the SLCs...
responded to progesterone and its ability to inhibit oxytocin signaling, as well as the time after removal of progesterone required for the SLCs and LLCs to respond to oxytocin and PGF$_{2\alpha}$ respectively, after removal of progesterone. Finally, alteration of membrane cholesterol content was examined as a potential mechanism for the inhibition of oxytocin and PGF$_{2\alpha}$ actions by progesterone.

**MATERIALS AND METHODS**

**Materials**

Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (West Sacramento, CA). Penicillin/streptomycin and Dulbecco modified Eagle medium (DMEM) were purchased from Mediatech, Inc. (Herndon, VA). Glass-bottom culture plates were purchased from MatTek Corp. (Ashland, MA). All steroid hormones, PGF$_{2\alpha}$, oxytocin, and methyl-$\beta$ cyclodextrin (M[CD]) were purchased from Sigma (St. Louis, MO). Fura-2 acetoxyethyl ester (Fura-2AM), Phenolic F-127, and the fluorescently labeled secondary antibodies were obtained from Molecular Probes (Invitrogen, Inc., Carlsbad, CA). The DNA-binding dye, 4,6-diamidino-2-phenylindole (DAPI), was purchased from Calbiochem (San Diego, CA). The antibody to the oxytocin receptor was purchased from Santa Cruz Biotechnology, Inc. (SC-8103; Santa Cruz, CA). The 1,2-3H]-cholesterol was purchased from Perkin Elmer (Waltham, MA). Matrigel was purchased from VWR International (West Chester, PA). All remaining chemicals were purchased from either Sigma or Fisher Scientific (Fairlawn, NJ). The antibody to $\beta$-hydroxysteroid dehydrogenase (HSD3B) was graciously provided by Dr. Ian Mason at the Centre for Reproductive Biology, Queen’s Medical Research Institute (Edinburgh, Scotland). Cholesterol-loaded cyclodextrin (CLC) was generously provided by Dr. James Graham at Colorado State University.

**Animals**

Western range ewes were used for all experiments. All surgical procedures were approved by Colorado State University Animal Care and Use Committee (protocol 04231A-02) and in compliance with National Institutes of Health guidelines.

**Luteal Cell Purification**

Corpora lutea were collected from two or three superovulated ewes on Day 9 or 10 of the estrous cycle [10], pooled, and dissociated into single-cell suspensions, followed by centrifugal elutriation to obtain partially purified small luteal cells (SLCs) and endothelial cells (LLCs). To determine cell-specific effects of oxytocin on the SLC types, luteal endothelial cells were separated from steroidogenic SLCs by a procedure described previously, with minor modification [13]. Magnetic toyrol-activated beads (4 x 10$^5$ beads; Dynal Biotech, Lake Success, NY) were incubated with BS-1 lectin (0.15 mg/ml; Vector Laboratories, Inc., Burlington, CA) for 24 h at 37°C with gentle rocking. The beads were washed with PBS and stored at 4°C. Cells in the SLC fraction (2 x 10$^6$) were mixed with 25 μl of BS-1 lectin-coated magnetic beads (1 x 10$^5$) and incubated for 30 min at 4°C with gentle rotation. The BS-1 lectin-coated beads bind to a specific carbohydrate on the cell surface of endothelial cells; therefore, when placed in front of a magnetic stand for 1 min, the endothelial cells migrate toward the magnet. The supernatant containing steroidogenic SLCs was transferred to a fresh microcentrifuge tube. The lectin-bound endothelial cell population was resuspended in PBS and placed in front of the magnet for further purification. Similarly, the steroidogenic SLC fraction collected was placed in front of the magnet to remove any remaining endothelial cells. Following separation, each cell type was resuspended in DMEM containing 100 IU/ml of penicillin, 50 μg/ml of streptomycin, and 10% FBS and plated overnight on chambered slides or Matrigel-coated 35-mm glass-bottom culture dishes.

**Immunocytochemistry**

To determine the purity of each isolated cell population, steroidogenic SLCs and endothelial cells were stained for the steroidogenic enzyme HSD3B. Cells were washed three times with PBS and incubated 10 min with 4% paraformaldehyde. Cells were washed after fixation and incubated with PBS containing 3% bovine serum albumin (BSA) for 1 h at room temperature to block nonspecific binding sites. Cells were incubated with a mouse anti-oxytocin antibody specific to the N-terminus (1 μg/ml) [14] for 1 h at room temperature, followed by an incubation with rabbit anti-goat Alexa 594 (1:500; Invitrogen, Inc., Molecular Probes, Carlsbad, CA) for 1 h at room temperature. Following the incubation with the secondary antibody, the cells were washed with PBS, and cell membranes were permeabilized with 2% paraformaldehyde containing 0.3% Triton X-100 for 10 min at room temperature. Cells were then incubated with the primary antibody anti-rabbit HSD3B (1:1000) and the secondary goat anti-rabbit Alexa 488 antibody using the same procedures as described for the anti-oxytocin receptor antibody. Substitution of the primary antibody with rabbit IgG (1 μg/ml) was used as a negative control. Last, cells were stained with DAPI (3 μg/ml) for visualization of the nuclei.

**Measurement of Intracellular Calcium**

After overnight incubation, cells were washed twice with fluorescence buffer (145 mM NaCl, 13 mM HEPES, 1 mM Na$_2$PO$_4$, 0.5 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM Hepes, and 5 mM glucose [pH 7.4]) and incubated at room temperature for 30 min with the calcium indicator Fura-2AM (3 μM) and 0.06% Phenolic F-127. Cells were washed twice with fluorescence buffer after Fura-2AM labeling and incubated for 30 min in fluorescence buffer. Changes in intracellular calcium concentrations in individual luteal cells were measured using an InCyt 2 imaging system (Intracellular Imaging, Inc., Cincinnati, OH) at 340 and 380-nm excitation and 510-nm emission wavelengths. Baseline intracellular calcium concentrations were obtained at 0.8-sec intervals for 30 sec before addition of either ethanol (0.1%), oxytocin (10 μM), or PGF$_{2\alpha}$ (1 μM). Measurement of intracellular calcium concentrations continued for 4 min 30 sec following treatment to determine the effects of the hormones on concentrations of intracellular calcium. Cells that had at least a 2-fold increase over baseline in the 340:380 fluorescence ratio were considered responsive to either oxytocin or PGF$_{2\alpha}$.

**Determination of Which Cell Type Within the SLC Fraction Responds to Oxytocin**

Small luteal cells were separated from luteal endothelial cells as already described. Small luteal cells (50000) or endothelial cells (50000) were plated on Matrigel-coated 35-mm glass-bottom culture dishes and incubated overnight in DMEM containing 10% FBS. Cells were treated with 10 μM oxytocin, and concentrations of intracellular calcium were measured. Changes in concentrations of intracellular calcium when cells were treated with oxytocin were determined using purified cells from three CL collections. Concentrations of intracellular calcium were measured for two dishes (15–50 cells/dish) per treatment per culture or CL preparation. Data are given as the average percentage of responsive cells for CL preparations (n = 3).

**Effects of Progesterone on Oxytocin-Stimulated Changes in Concentrations of Intracellular Calcium in SLCs**

Three individual experiments were conducted to determine the effects of progesterone on oxytocin-induced changes in intracellular concentrations of calcium in SLCs. In the first experiment, the duration of progesterone exposure required before SLCs would become unresponsive to the effects of oxytocin was determined. Progesterone (95 μM) was added to SLCs for 15 min, 1 h, 3 h, 6 h, or >16 h. With the progesterone remaining within the fluorescence buffer, oxytocin (10 μM) was added to the cells, and changes in concentrations of intracellular calcium were measured as already described. Concentrations of intracellular calcium were measured for two dishes (15–40 cells/dish) per treatment per culture or CL preparation. Data are given as the average percentage of responsive cells for CL preparations (n = 3 for 15 min and n = 5 for >16 h). In the second experiment, we determined if progesterone had acute effects on concentrations of intracellular calcium in SLCs. Small luteal cells were treated with progesterone (95 μM) for 15 min, and concentrations of intracellular calcium were measured. After the 15-min exposure to progesterone, oxytocin (10 μM) was added to the cells, and changes in intracellular calcium concentrations were measured for an additional 6 min. Concentrations of intracellular calcium were measured for two dishes (15–58 cells/dish) per treatment per culture or CL preparation; cultures were replicated three times and averaged. For the third experiment, we wanted to determine when SLCs became responsive to oxytocin following removal of progesterone. At the time of plating, cells were treated with 95 μM progesterone until 1, 3, or 6 h before measurement of concentrations of intracellular calcium. The cells in the remaining treatment group were not treated with progesterone at the time of plating (>16 h). Changes in concentrations of intracellular calcium following oxytocin treatment (10 μM) were measured as described previously. Concentrations of intracellular calcium...
were measured for two dishes (10–30 cells/dish) per treatment per culture or CL preparation. Cultures were replicated six times, and data are shown as the average percentage of responsive cells.

Effects of Progesterone on PGF$_{2\alpha}$-Stimulated Changes in Concentrations of Intracellular Calcium in LLCs

We have shown that 95 μM of progesterone inhibited the ability of PGF$_{2\alpha}$ to increase concentrations of intracellular calcium concentrations; however, the inhibition was inconsistent [9]. It was speculated that high basal secretion of progesterone from the LLCs in culture might be contributing to the inconsistencies observed. To address this issue, LLCs were treated with 0.2 mM aminoglutethimide [15] to inhibit progesterone production. We wanted to determine when LLCs would become responsive to PGF$_{2\alpha}$ after removal of progesterone. At the time of plating, LLCs were treated with 0.2 mM aminoglutethimide and 95 μM progesterone until 1, 3, or 6 h before measurement of intracellular calcium concentrations. Cells in the remaining treatment group (>16 h) were treated with 0.2 mM aminoglutethimide but did not receive progesterone. Changes in concentrations of intracellular calcium were measured after PGF$_{2\alpha}$ treatment for two dishes (12–30 cells/dish) per culture or CL preparation as already described. Cultures were replicated three times, and data are given as the average percentage of responsive cells.

Alteration of Membrane Cholesterol Content in SLCs and LLCs

It has been shown that micromolar concentrations of progesterone alter membrane cholesterol content [16]. To determine if the suppressive effect of progesterone on the actions of oxytocin could be mimicked, cholesterol content in the plasma membrane of SLCs was altered using cyclodextrins. An initial study was conducted using radiolabeled cholesterol to determine if treatment of SLCs with MβCD did indeed remove cholesterol from the plasma membranes of SLCs. The [3H]cholesterol was bound to high-density lipoprotein (HDL) as described by Brown et al. [17]. Briefly, 100 μl (approximately 5.4 × 10$^3$ cpm) of [3H]cholesterol was mixed with an equal volume of chloroform:methanol (v:v, 2:1), and the chloroform:methanol mixture was evaporated. To the [3H]cholesterol, 0.5 ml of solution (0.15 M NaCl, 0.3 mM ethylenediamine tetraacetic acid (EDTA), and 10% dimethyl sulfoxide (DMSO) (pH 7.0)) was added and incubated for 10 min at 37°C, after which 10 μl of HDL (112 μg of protein) was added. The [3H]cholesterol and HDL mixture was incubated for 4 h at 37°C. Small luteal cells were incubated with [3H]cholesterol-HDL (2 × 10$^3$ cpm) for 16–18 h at 37°C to allow cellular uptake of the [3H]cholesterol-HDL. Media were removed, and cells were washed with PBS. Fresh media containing progesterone (95 μM) or MβCD (1 or 10 mM) were added to the cells for 1 h at 37°C. These are similar concentrations of MβCD reported to alter cholesterol content of the plasma membrane and subsequent oxytocin binding and function of the oxytocin receptor [18, 19]. Media were removed and transferred to a vial containing scintillation fluid, and the amount of radioactivity in the culture media was determined using a β-scintillation counter.

To determine if removal of cholesterol from the plasma membrane altered the ability of oxytocin and PGF$_{2\alpha}$ to increase concentrations of intracellular calcium in SLCs and LLCs, respectively, cells were treated for 40–60 min with 1 mM MβCD. Cells were then treated with 10 μM oxytocin (SLCs) or 1 μM PGF$_{2\alpha}$ (LLCs), and changes in concentrations of intracellular calcium were measured. In an additional treatment group, SLCs or LLCs were treated for >16 h with 95 μM progesterone. These cells were treated with 0.5 mg/ml of CLC for 40–60 min in the presence of progesterone to replenish cholesterol within the plasma membranes. Cells were treated with oxytocin or PGF$_{2\alpha}$, and concentrations of intracellular calcium were measured for two dishes of cells per CL preparation (n = 3). Data are given as percentage cells responsive as average values for three CL collections.

Statistical Analysis

All statistical analyses were performed with the general linear model procedure of SAS (version 9.1; Statistical Analysis System Institute, Cary, NC) [20]. Differences among treatment means were detected using least significant difference test or Tukey honestly significant difference test.

RESULTS

In our previous study [9], it was shown the cells in the SLC fraction responded to oxytocin in a dose-dependent manner. Because this cell fraction contains both SLCs and endothelial cells [10] and it was not known if the cells that responded to oxytocin with an increase in concentrations of intracellular calcium were the steroidogenic cells, we removed the endothelial cells using BS-1 lectin-coated magnetic beads. As determined by staining for HSD3B, an almost pure population of small steroidogenic luteal cells was obtained following the separation procedure (Fig. 1, A and B). A few SLCs were observed in the isolated endothelial cell population (Fig. 1, C and D). When the two cell populations were stained for oxytocin receptors, only steroidogenic SLCs contained oxytocin receptors (Fig. 2), and then only a mean ± SEM of 47.4%...
± 6.6% of the steroidogenic SLCs were stained positive for the oxytocin receptor. Staining for oxytocin receptors was not observed for endothelial cells (data not shown). To confirm the immunohistochemical data, each cell type was also treated with oxytocin, and changes in concentrations of intracellular calcium were measured. A significantly greater mean ± SEM percentage of SLCs (52.9% ± 10.9%) responded with an increase in concentrations of intracellular calcium compared with endothelial cells (12.3% ± 3.4%, P < 0.05). The percentage of SLCs responsive to oxytocin is similar to the percentage of cells stained for oxytocin receptors.

Cells were treated for 15 min, 1 h, 3 h, 6 h, or >16 h with progesterone (95 μM) to examine the effects of progesterone on the ability of SLCs to respond to oxytocin. The percentage of cells that exhibited an increase in concentrations of intracellular calcium when treated with oxytocin (10 μM) was decreased by 1 h of progesterone treatment and remained decreased at 3, 6, and >16 h (Fig. 3, P < 0.05). Furthermore, progesterone (95 μM) does not have an acute effect on concentrations of intracellular calcium as shown by the lack of change in the mean ± SEM fluorescence 340:380 ratio when cells were treated for 15 min with progesterone (0.74 ± 0.09) or DMSO (0.65 ± 0.04).

To further investigate the effects of progesterone on the actions of oxytocin, a time course of progesterone removal was conducted. There was a significant increase in the percentage of SLCs responding to oxytocin in as little as 1 h after progesterone removal, with a further increase at 6 h, and the responsiveness was maintained through 16 h (Fig. 4, P < 0.05). Progesterone also appeared to affect the actions of progesterone removal on the percentage of SLCs (solid bars) responsive to oxytocin or LLCs (dashed bars) to PGF2α. Progesterone (95 µM) was removed 0, 1, 3, 6, or >16 h before treatment with oxytocin (10 µM) or PGF2α (1 µM), and concentrations of intracellular calcium were measured. Values are the mean ± SEM for six CL preparations for SLCs and for three CL preparations for LLCs. Different letters denote significant differences (P < 0.05).
PGF\(_{2\alpha}\) on LLCs. The percentage of LLCs responding to PGF\(_{2\alpha}\) increased after 3 h of progesterone removal, and a similar responsiveness was maintained through 6 and >16 h of progesterone removal (Fig. 4, \(P < 0.05\)).

The ability of cyclodextrins to mimic the effects of progesterone and inhibit the ability of oxytocin and PGF\(_{2\alpha}\) to alter concentrations of intracellular calcium in steroidogenic luteal cells was examined. To confirm that MβCD removed cholesterol from the plasma membrane, SLCs that had been provided with \(^{3}H\)cholesterol-HDL were treated with MβCD, and culture media were analyzed. Indeed, the mean ± SEM amount of \(^{3}H\)cholesterol measured in the culture media was greater (\(P < 0.05\)) in cells treated with 1 mM MβCD (33498 ± 4119 cpm) or 10 mM MβCD (140359 ± 20852 cpm) than vehicle (6275 ± 2746 cpm) or 95 μM progesterone (10367 ± 1322 cpm). Cell viability was reduced with 10 mM MβCD; therefore, 1 mM MβCD was used to determine the effect of reduced membrane cholesterol content on the ability of oxytocin or PGF\(_{2\alpha}\) to increase concentrations of intracellular calcium. Addition of 1 mM MβCD decreased (Fig. 5, \(P < 0.05\)) the percentage of SLCs responsive to oxytocin to a number similar to that observed for 95 μM progesterone (>16 h). It was speculated that under the current culture conditions CLC (0.5 mg) replenished cholesterol within the plasma membrane of luteal cells. Addition of CLC to SLCs treated with progesterone prevented the inhibitory effects of progesterone on oxytocin-stimulated increase in concentrations of intracellular calcium (Fig. 5). Similar effects were observed with LLCs treated with the cyclodextrins, although the decrease in percentage of LLCs responsive to PGF\(_{2\alpha}\) was not as great with MβCD treatment as with progesterone (Fig. 5).

**DISCUSSION**

From these experiments, we suggest that ovine steroidogenic SLCs contain functional oxytocin receptors. Oxytocin-binding sites have been identified in cultured bovine (Kd = 0.2 nM) [21] and porcine (Kd = 0.2 nM) luteal cells [22] collected during early, mid, and late luteal phases. Oxytocin induced a rapid and transient increase in intracellular concentrations of calcium in SLCs that appeared to be due to release of calcium from intracellular stores (data not shown). Oxytocin receptors in the myometrium are coupled to G\(_{q/11}\) proteins that stimulate the activity of phospholipase C\(_{q}\) generation of inositol triphosphate (IP\(_{3}\)), and release of calcium from intracellular stores [23]. Not all of the SLCs exhibited an increase in intracellular concentrations of calcium when treated with oxytocin. There are two possible explanations for the lack of response of the entire population of SLCs to oxytocin. It has been suggested that a threshold of the intracellular signal must be obtained to PGF\(_{2\alpha}\) in LLCs before intracellular calcium concentrations will increase [24]. The more likely explanation for the lack of response is that the cells that did not respond did not contain oxytocin receptors, as it was shown by immunohistochemistry that not all steroidogenic SLCs contained detectable numbers of oxytocin receptors. It remains unclear why the entire population of SLCs does not have detectable numbers of oxytocin receptors.

In the present study, progesterone inhibited the ability of oxytocin and PGF\(_{2\alpha}\) to increase intracellular calcium concentrations in ovine steroidogenic SLCs and LLCs, respectively. Although this is the first report of progesterone inhibiting the actions of PGF\(_{2\alpha}\), other laboratories have reported that progesterone inhibits the actions of oxytocin in cell lines and endometrial tissue. Progesterone inhibits production of IP3 in ovine endometrial explants [6], Chinese hamster ovary (CHO) cells [25], and COS-7 cells [26], as well as increases in intracellular concentrations of calcium in endometrial cells [27,28]. Progesterone also inhibits oxytocin-stimulated PGF\(_{2\alpha}\) secretion from ruminant endometrium at high molar concentrations (10 – 5 M) [24, 25] and at more physiological concentrations (16 nM) [6].

The suppressive effect of progesterone on the actions of oxytocin and PGF\(_{2\alpha}\) on steroidogenic luteal cells is likely a nongenomic action, although it appears that the mechanism by which progesterone blocks the actions of oxytocin in the CL differs from what has been reported in endometrial tissue. Progesterone inhibits binding of oxytocin to its receptor in ovine [6, 29] and bovine [27] endometrial plasma membranes and CHO cells transfected with the rat oxytocin receptor [25]. There is evidence that progesterone inhibits oxytocin binding by directly competing with oxytocin for the oxytocin receptor or with proteins associated with the oxytocin receptor in ovine endometrial plasma membranes [6]. In contrast, the inhibitory effects of progesterone on the stimulation of inositol phosphate hydrolysis by oxytocin in COS-7 cells do not appear to be mediated via progesterone acting through either the progesterone or oxytocin receptor, as these cells contain little to no nuclear progesterone receptors, and no specific binding of a synthetic progesterone was detected in the COS-7 cells transfected with the ovine oxytocin receptor [26].

In contrast to the previous experiments in which progesterone treatments were similar to concentrations in circulation (nanomolar concentrations), we used concentrations similar to intraluteal concentrations (micromolar concentrations) based on the progesterone concentrations (10–30 μg/g of tissue) in ovine luteal tissue collected during the midluteal phase [30]. Burger et al. [7] reported that concentrations of progesterone (10–200 μM) similar to those used in the present study abolished oxytocin signaling in human embryonic kidney cells transfected with the oxytocin receptor. In contrast to the reports that progesterone competes with oxytocin for the oxytocin receptors, Burger et al. [7] suggest that microgram concentra-
tions of progesterone alter cholesterol content in caveolae, which disrupts G protein-coupled receptor signaling. Indeed, microgram concentrations of progesterone prevent cholesterol movement from the endoplasmic reticulum to caveolae in the plasma membrane [16], where oxytocin receptors are predominantly localized [31, 32]. A reduction of the concentration of cholesterol in membranes lowers the affinity of oxytocin for its receptor [18, 33]. Furthermore, the effect may be mediated directly at the plasma membrane. Wenz and Barrantes [8] reported that progesterone at micromolar concentrations can disrupt the lipid domains in artificial membrane bilayers. Based on the data from the present study, progesterone appears to alter the content of cholesterol in the plasma membrane of luteal cells, thereby in some way rendering the receptors for oxytocin and PGF₂α unable to either bind to or signal through these receptors. Indeed, when SLCs and LLCs were treated with MTPCD to remove cholesterol from the plasma membrane, the response to oxytocin and PGF₂α was similar to that when the cells were treated with progesterone. The strongest evidence that cholesterol is altered by progesterone was shown when (even in the presence of progesterone) exposure to CLC and (assuming under the current culture conditions) replenishment of cholesterol reversed the inhibitory effects of progesterone in both SLCs and LLCs. Finally, the ability to inhibit cell signaling by oxytocin and PGF₂α is specific to progesterone and no other steroid hormones [9]. This is in agreement with Wenz and Barrantes [8] that of the steroid hormones progesterone has more lipid domain-disrupting activity than other steroid hormones.

In conclusion, progesterone alters the membrane of luteal cells such that cell signaling via membrane receptors is hindered. We have shown that progesterone inhibits oxytocin and PGF₂α signaling in ovine SLCs and LLCs, respectively. The percentage of SLCs and LLCs responsive to oxytocin and PGF₂α, respectively, begins to increase within an hour of progesterone removal, with a significant increase by 3 h of progesterone removal. From the present data, we hypothesize that at the time of luteolysis a few LLCs are responsive to the initial pulse release of PGF₂α from the uterus. The initial pulse of PGF₂α binds to and activates the receptors on the few responsive LLCs to initiate the decline in progesterone production. As intraluteal concentrations of progesterone decline over time, more cells become responsive to both oxytocin and PGF₂α, as indicated by the increase in intracellular concentrations of calcium. The role of the increases in intracellular calcium induced by oxytocin and PGF₂α in luteal cells is not known; however, because both hormones are involved in luteolysis, it seems likely that the increases in calcium are involved in apoptosis of the luteal cells during luteal regression.

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