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Developmental Sensitivity of the Bovine Corpus Luteum to Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and Endothelin-1 (ET-1): Is ET-1 a Mediator of the Luteolytic Actions of $PGF_{2\alpha}$ or a Tonic Inhibitor of Progesterone Secretion?[1]

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ABSTRACT

We examined the responsiveness of large luteal cells (LLC), small luteal cells (SLC), and endothelial cells of the Day 4 and Day 10 bovine corpus luteum (CL) to prostaglandin (PG) $F_{2\alpha}$ and endothelin (ET)-1. Using a single-cell approach, we tested the ability of each agonist to increase the cytoplasmic concentration of calcium ions ($[Ca^{2+}]_i$) as function of luteal development. All tested concentrations of agonists significantly ($P = 0.05$) increased $[Ca^{2+}]_i$ in all cell populations isolated from Day 4 and Day 10 CL. Day 10 steroidogenic cells were more responsive than Day 4 cells to $PGF_{2\alpha}$ and ET-1. Response amplitudes and number of responding cells were affected significantly by agonist concentration, luteal development, and cell type. Response amplitudes were greater in LLC than in SLC; responses of maximal amplitude were elicited with lower agonist concentrations in Day 10 cells than in Day 4 cells. Furthermore, on Day 10, as the concentration of $PGF_{2\alpha}$ increased, larger percentages of SLC responded. Endothelial cells responded maximally, regardless of agonist concentration and luteal development. In experiment 2, we tested the developmental responsiveness of total dispersed and steroidogenic-enriched cells to the inhibitory actions of $PGF_{2\alpha}$ and ET-1 on basal and LH-stimulated progesterone accumulation. The potency of $PGF_{2\alpha}$ steroidogenic-enriched cells on Day 4 was lower than on Day 10; in contrast, the potency of ET-1 was not different. Therefore, ET-1 was a tonic inhibitor of progesterone accumulation rather than a mediator of $PGF_{2\alpha}$ action. The lower efficacy of $PGF_{2\alpha}$ in the early CL more likely is related to signal transduction differences associated with its receptor at these two developmental stages than to the inability of $PGF_{2\alpha}$ to up-regulate ET-1.

calcium, corpus luteum function, ovary, progesterone, signal transduction

INTRODUCTION

The corpus luteum (CL) is a transient gland that is necessary for maintenance of pregnancy, and its regression is essential for normal ovarian cyclicity. In the majority of species studied, prostaglandin (PG) $F_{2\alpha}$ is the primary factor responsible for cessation of luteal progesterone (P_4) production and luteal cell involution. This luteolytic process involves interactions between at least three luteal cell populations: endothelial cells, and the two steroidogenic cells (large luteal cells [LLC] and small luteal cells [SLC]). Studies to understand luteal physiology have devoted greater attention to the steroidogenic than to the endothelial cell population. Yet, endothelial cells contribute approximately 50% of the total cell population of the CL [1]. They are known to express the $PGF_{2\alpha}$ receptor [2], and $PGF_{2\alpha}$ has been reported to have direct effects on bovine luteal endothelial cells [3].

In vitro studies examining direct effects of $PGF_{2\alpha}$ on P_4 production by luteal steroidogenic cells have produced inconsistent results [4, 5], and these results have not always agreed with those of in vivo studies. One possible reason for the discrepancy between the in vivo and in vitro results is that the actions of $PGF_{2\alpha}$ are not exerted directly on steroidogenic cells but, rather, are mediated by the luteal endothelial cells [5]. A large body of data supports the notion that endothelin (ET)-1, secreted by luteal endothelial cells, plays an essential role during luteolysis. It has been hypothesized that the luteal role of ET-1 might be to mediate the luteolytic actions of $PGF_{2\alpha}$ [3, 6–13].

In cows and various other animals in which $PGF_{2\alpha}$ induces luteolysis, the CL of the early estrous cycle (Days 1–5) is resistant to the luteolytic action of a dose of $PGF_{2\alpha}$ that induces luteolysis in mid to late CL (Days 8–15). The mechanisms responsible for this insensitivity of the early CL to $PGF_{2\alpha}$ are not fully understood; however, several possibilities have been implicated [14–16]. In the context of ET-1 as a mediator of the luteolytic actions of $PGF_{2\alpha}$, an additional mechanism that might participate in the resistance of the early CL has been reported [6]. It has been proposed that because the luteolytic actions of $PGF_{2\alpha}$ require the intermediary role of ET-1 and administration of $PGF_{2\alpha}$ can induce ET-1 gene expression only during the late luteal phase, it is reasoned that during the early luteal phase, the CL is insensitive to the luteolytic actions of $PGF_{2\alpha}$. However, it has been demonstrated recently that although an injection of $PGF_{2\alpha}$ does not up-regulate ET-1 synthesis in the early CL, the local regulation of the entire luteal ET system occurs in such a manner that a steady increase occurs in the luteal content of ET-1 from Day 1 [17]. More importantly, the amount of mature ET-1 peptide in Day 4 and Day 10 CL did not differ [17]. These latter observations raise the possibility that if the early CL has the capacity to respond to the available ET-1, then the luteal role for ET-1 is unlikely to be mediation of the luteolytic

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actions of $PGF_{2\alpha}$. The test of this hypothesis would require examining the sensitivity of the CL to ET-1 and $PGF_{2\alpha}$ as a function of luteal development. To our knowledge, no studies have examined these three cell populations in vitro to determine their sensitivity to $PGF_{2\alpha}$ and ET-1 as a function of luteal development. The urgency of including luteal endothelial cells in studies to understand the mechanisms of luteolytic sensitivity in the CL is accentuated by the observation that one of the direct actions of $PGF_{2\alpha}$ on luteal endothelial cells of the Day 10 CL is to modulate the expression of several genes of the ET system [5].

In luteal steroidogenic cells, $PGF_{2\alpha}$ activates its plasma membrane G protein-coupled receptor. Some controversy exists regarding the steroidogenic cell type expressing these receptors; it appears that in the cow, both LLC and SLC express the receptor, with LLC having a higher expression [2, 3, 18]. Nevertheless, $PGF_{2\alpha}$, binding to its cognate receptor in the steroidogenic cells, activates the membrane-bound, phosphoinositide-specific phospholipase (PL) C, yielding inositol 1,4,5-trisphosphate and diacylglycerol [19]. Indeed, in bovine luteal cells, $PGF_{2\alpha}$ stimulates phosphatidylinositol 4,5-biphosphate hydrolysis and mobilizes intracellular Ca^{2+} [20, 21]. Accordingly, calcium and protein kinase (PK) C have been shown to be the intracellular mediators of actions of $PGF_{2\alpha}$ in luteal cells [22]. Despite the fact that direct actions of $PGF_{2\alpha}$ in luteal endothelial cells have been reported, to our knowledge no studies have addressed the relationship between the intracellular mediators of actions of $PGF_{2\alpha}$ in endothelial luteal cells. The possibility that intracellular signal transduction mechanisms might contribute to the insensitivity or resistance of the early CL has not been explored in the whole CL or in any individual luteal cell population.

Here, we report the results of two experiments designed to test the hypothesis that ET-1 is a mediator of the luteolytic actions of $PGF_{2\alpha}$ and that it might participate in the resistance of the early CL. Specifically, we have investigated the sensitivity of these three luteal cell populations, from the point of view of signal transduction, to $PGF_{2\alpha}$ and ET-1 as a function of luteal development (Day 4 vs. Day 10). The sensitivity of total dispersed (a mixture of steroidogenic and endothelial luteal cells) and steroidogenic-enriched luteal cells to the inhibitory actions of $PGF_{2\alpha}$ and ET-1 on basal and LH-stimulated P_4 accumulation also was examined at these two developmental stages. The answers to the questions posed in the present studies are important, because they will help us to understand as well as fine-tune the luteal role of ET-1 and the mechanisms participating in the resistance of the early CL to the luteolytic actions of $PGF_{2\alpha}$.

MATERIAL AND METHODS

CL Collection

Non-lactating beef cows were observed visually for estrus twice daily at approximately 12-h intervals for a minimum of 30 min per observation. The day when standing estrus was observed was designated as Day 0 [22]. After two cycles, Day 4 and Day 10 CL were collected by blunt dissection (Day 10) or ovariectomy (Day 4) via supravaginal incision under epidural anesthesia. For the epidural anesthesia, 6–9 ml of 2% lidocaine were administered to cows weighing 450–700 kg (Butler Company, Columbus, OH). The CL or ovary was collected into ice-cold PBS (pH 7.4) and transported to the laboratory within 15–30 min after collection. This investigation was conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching. The West Virginia University Animal Care and Use Committee reviewed and approved the protocol for the tissue collection (ACUC no. 01-0809).

Luteal Cell Dispersion and Purification

In the laboratory, the CLs were dissected free of connective tissue, weighed, placed in cell dispersion medium (CDM; medium 199 [M199] containing 0.1% BSA, 25 mM Hepes, and 100 U/ml of fungicide), and cut into small (~1 mm^3) fragments. The tissue fragments were washed twice with CDM and placed into 5 ml of fresh CDM containing collagenase type IV (420 U/ml/g tissue; Gibco, Invitrogen Life Technologies, Carlsbad, CA). After 1 h of incubation at 35°C in a shaking (200-rpm) water bath (New Brunswick Scientific, Edison, NJ), the digestion mixture was aspirated several times with a Pasteur pipette to dissociate the tissue mechanically. Undigested tissue was allowed to settle, and the supernatant was transferred to a 50-ml, sterile plastic tube to collect the cells by centrifugation (100 × *g* for 5 min). The dissociated cells were washed twice with CDM and placed on ice. The undigested tissue was returned to the shaking water bath in fresh CDM/collagenase. This procedure was repeated until all the tissue had been digested. The pooled dispersed luteal cells were filtered through a sterile nylon membrane to remove any remaining tissue debris. Luteal endothelial cells were separated by a procedure described previously [12]. Briefly, magnetic tosylactivated beads (Dynal Biotech, Lake Success, NY) were coated with BS-1 lectin (0.15 mg/ml; Vector Laboratories, Inc, Burlingame, CA) for 24 h at room temperature. The beads were washed and stored at 4°C until use. Dispersed luteal cells were suspended in 1% PBS, mixed with beads at a bead: endothelial cell ratio of 1:3, and placed for 25 min at 4°C on a rocking platform. The bead-adherent cells were washed with 1% PBS and concentrated using a magnetic particle concentrator (Dynal Biotech, Lake Success, NY). Both BS-1-adhering (endothelial cells) and nonadherent cells (steroidogenic-enriched luteal cells) were collected by this procedure. In the present study, all cells in the population that we call endothelial cells had beads attached, but the cell purity of the fraction was not characterized further. Similarly, the cell population that we call steroidogenic cells did not have beads attached, but they represented a heterogeneous population of cells, including fibroblasts, pericytes, lymphoid, and any endothelial cells not removed by our separation procedure. Cell viability and density were determined using trypan blue exclusion and a hemocytometer; luteal cell viability was greater than 96%.

Single-Cell Calcium Measurements

The cell density of the enriched populations of luteal cells was adjusted to 1×10^5 cells/ml by adding bicarbonate-buffered M199 supplemented with 5.0% fetal calf serum (FCS). This initial concentration of FCS in the M199 allowed luteal cell attachment to the microscope slides. A 60-μl aliquot of the cell suspension was applied to a Cunningham chamber constructed on poly-L-lysine-coated microscope slides [23, 24]. The Cunningham chambers were maintained overnight in a tissue-culture incubator (37°C, 95% air/5% CO_2). Poly-L-lysine, M199, FCS, and penicillin-streptomycin were from Life Technologies (Grand Island, NY).

The following day, the cells were used for calcium measurements. The tissue-culture media in these experiments consisted of 127 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM $KHPO_4$, 5 mM $NaHCO_3$, 10 mM Hepes, 10 mM glucose, and 0.1% BSA at pH 7.4. Luteal cells were loaded with 1 μM fura-2/AM (Calbiochem, San Diego, CA) in experimental medium (without hormones) for 20 min at 37°C. The cells were washed with experimental medium and incubated for an additional 20 min at 37°C to allow cytoplasmic de-esterification of the fura-2/AM dye.

After dye loading, the Cunningham chamber was placed on the stage of an Olympus PROVIS AX70 microscope (Olympus America, Inc., Melville, NY) equipped for epifluorescence microscopy. All experiments were performed at room temperature (22–25°C). The excitation light was supplied by a mercury short arc photo-optic lamp source (OSRAM; B&B Microscopes, Warrendale, PA). The excitation wavelengths were selected by 340- and 380-nm filters (half-bandwidth, 2 nm; Chroma Technology Corporation, Brattleboro, VT) mounted in a rotating filter wheel (MAC 2000; Ludl Electronic Products, Hawthorne, NY) between the mercury lamp and the microscope. The rotating filter wheel was operated by a LAMDA 10-2 controller (Scanalytic, Inc., Fairfax, VA). This automated shutter and filter controller allowed acquisition of images for dual-wavelength microscopy. Fluorescence images were collected via the objective lens and passed through a barrier filter (transmission wavelength, 490–600 nm) to the face of a PVCam camera (Photometric SenSys, Michigan City, IN). These images were acquired and analyzed using IPLAB ratio software for the Macintosh computer (Version 3.2; Scanalytic, Inc., Fairfax, VA). For further analysis, the cell responses were represented as changes in the 340/380-nm fluorescence ratio over time. Changes in fluorescence ratio at these two wavelengths have been demonstrated to be

caused by changes in $[Ca^{2+}]_i$. Microscopic fields were selected using a bright-field image where cell size and cell morphology could be determined. Both steroidogenic cells were round, with SLC being less than 20 μm in diameter and LLC being greater than 20 μm in diameter. The endothelial cells were flat, spindle shaped, and had one or two magnetic beads attached. This field-selection procedure allowed recording of two to three cells per slide.

Experiment 1

Experiment 1 examined developmental sensitivity of luteal cells to $PGF_{2\alpha}$, and ET-1. Specifically, single-cell studies were conducted to compare the ability of $PGF_{2\alpha}$ and ET-1 to elicit $[Ca^{2+}]_i$ responses by luteal steroidogenic and endothelial cells isolated from Day 4 and Day 10 CL. The ability of different concentrations of agonists ($PGF_{2\alpha}$: 0, 10, 100, and 1000 ng/ml; ET-1: 0, 10, 100, and 1000 nM) to stimulate a rise in $[Ca^{2+}]_i$ was examined using a protocol previously described for porcine granulosa cells [25]. To our knowledge, the cellular effector system activated by the ET receptor in bovine luteal cells has not been studied. However, in porcine granulosa cells, the ET receptors have been shown to activate the PLC effector system [25, 26]; therefore, we expected these receptors to use a signal transduction mechanism similar to that in bovine luteal cells. Each agonist concentration was evaluated in three slides per experimental animal. At least six independent replicates (experimental animals) were performed for each developmental stage of the CL. This experiment tested the ability of two agonists to stimulate a rise in $[Ca^{2+}]_i$ as a function of the developmental stage of the CL and the concentration of agonist. At least two variables were examined: the fold-increase of the elicited change in fluorescence ratio, and the percentage of cells responding to each agonist concentration.

Experiment 2

Experiment 2 also examined the developmental sensitivity of luteal cells to $PGF_{2\alpha}$ and ET-1. Specifically, responsiveness of Day 4 and Day 10 luteal cells to varying concentrations of $PGF_{2\alpha}$ (0, 10, 100, and 1000 ng/ml) or ET-1 (0, 10, 100, and 1000 nM) was tested by measuring the basal and LH (100 ng/ml)-stimulated P_4 accumulated during a 4-h interval in cultures of total dispersed and steroidogenic cells. Preliminary observations had indicated that LH at the concentration used here was effective in stimulating P_4 accumulation in luteal cells obtained from Day 4 and Day 10 CL. The total dispersed and steroidogenic cells were cultured in 24-well plates (1×10^3 steroidogenic cells/well). The cells were added in small aliquots to wells containing 1 ml of M199 with increasing concentration of $PGF_{2\alpha}$ and ET-1. The cells were incubated for 4 h at 37°C (95% air, 5% CO_2). After incubation, cell-free medium was removed from each well and frozen until assayed for measurement of P_4. Measurements of P_4 in the culture media were performed using an RIA as described previously [27]. The standard curve for this RIA ranged from 10 to 800 pg/ml, and the intra- and interassay coefficients of variation were 9.2% and 12.8%, respectively.

Statistical Analysis

Statistical analyses were performed using JMP 3.0, a statistical software program from Statistical Analysis System [28]. Data are presented as the mean ± SEM for all experiments. The data for fold-increase (340/380-nm ratio) and P_4 concentration were log-transformed to meet the assumptions of normality, and for data presentation, all the means were back-transformed accordingly. Two-way ANOVA was used to determine effects of different treatments. The Tukey-Kramer honestly significant difference test was used to compare the different treatments. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Experiment 1: $[Ca^{2+}]_i$ Responses

All tested concentrations of $PGF_{2\alpha}$ and ET-1 elicited significant ($P < 0.05$) and agonist-specific elevations in $[Ca^{2+}]_i$ in both populations of steroidogenic cells (LLC and SLC) isolated from Day 4 (not shown) and Day 10 CL (Figs. 1 and 2). Agonist specificity of the response was demonstrated by the observation that changes in the fluorescence ratio in cells challenged with media alone (top of Figs. 1 and 2) were of lower magnitude than those associated with an agonist challenge (middle $[PGF_{2\alpha}]$ and bottom [ET-1] of Figs. 1 and 2). Representative traces of the elevations in $[Ca^{2+}]_i$ observed in LLC (Fig. 1) and SLC (Fig. 2) isolated from a Day 10 CL when challenged with media (top, Fig. 2), $PGF_{2\alpha}$ (1000 ng/ml) (middle, Figs. 1 and 2), and ET-1 (100 nM) (bottom, Figs. 1 and 2) are shown. Traces obtained from Day 4 cells were similar and are not shown. The agonists typically stimulated greater fold-increases in $[Ca^{2+}]_i$ in LLC ($P = 0.03$) than in SLC (Figs. 1 and 2).

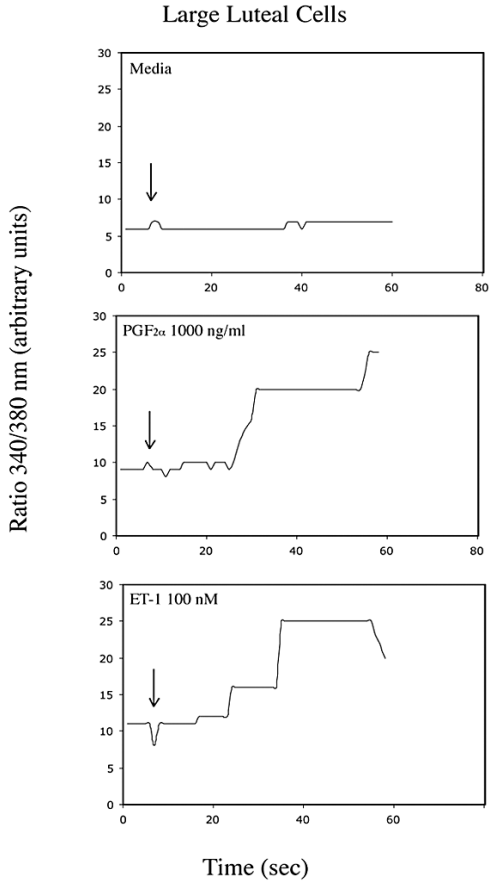

FIG. 1. Representative traces to illustrate the ability of $PGF_{2\alpha}$ (1000 ng/ml) and ET-1 (100 nM) to evoke a specific rise in the intracellular concentration of Ca^{2+} in single LLC isolated from Day 10 bovine CL. The cells were isolated and prepared for fura-2/AM imaging of $[Ca^{2+}]_i$ as described in *Materials and Methods.* Data are the relative fluorescence ratio (340/380 nm) over time (sec). Cells were exposed at the indicated time (arrows) to vehicle media (top), media containing 1000 ng/ml of $PGF_{2\alpha}$ (middle), and media containing 100 nM ET-1 (bottom).

Day 4 CL. In steroidogenic cells isolated from Day 4 CL, a dose-dependent effect of $PGF_{2\alpha}$ on the fold-increase of the elicited rise in $[Ca^{2+}]_i$ was observed only in the LLC population. The fold-increases in $[Ca^{2+}]_i$ stimulated by $PGF_{2\alpha}$ at the concentrations of 10 and 100 ng/ml were greater than those observed with media alone (Fig. 3A) but were not different from each other ($P = 0.29$). The fold-increase of the Ca^{2+} transients elicited by $PGF_{2\alpha}$ at the concentration of 1000 ng/ml were greater than those elicited at lower concentrations (Fig. 3A).

The percentage of Day 4 LLC responding to a challenge with 10 ng/ml $PGF_{2\alpha}$ was greater than that of those challenged with media alone (70% ± 2.3% vs. 6.3% ± 4.5%), and this percentage was not increased ($P = 0.34$) as the cells were challenged with greater concentrations of $PGF_{2\alpha}$ (Table 1).

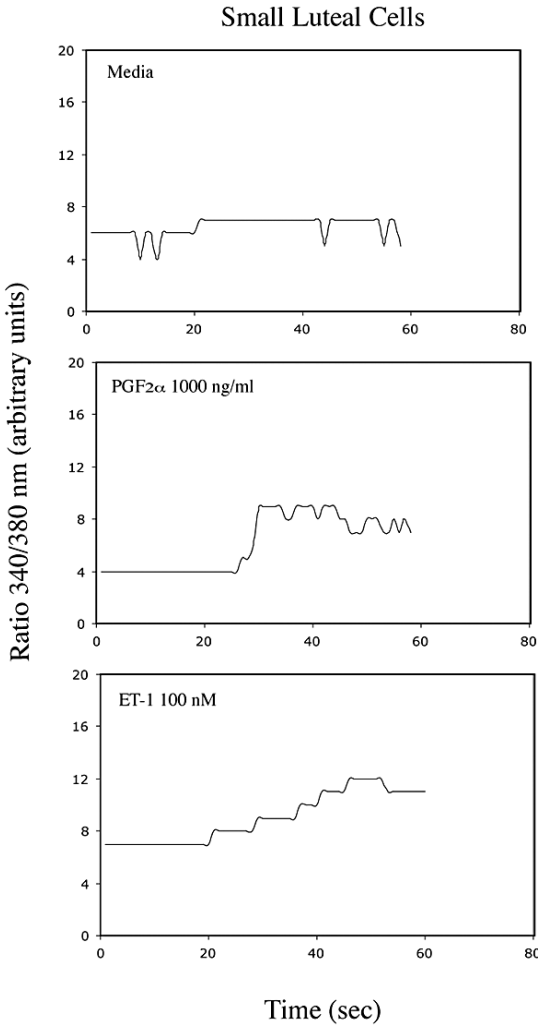

FIG. 2. Representative traces to illustrate the ability of $PGF_{2\alpha}$ (1000 ng/ml) and ET-1 (100 nM) to evoke a specific rise in the intracellular concentration of Ca^{2+} in single SLC isolated from Day 10 bovine CL. The cells were isolated and prepared for fura-2/AM imaging of $[Ca^{2+}]_i$ as described in *Materials and Methods*. Data are the relative fluorescence ratio (340/380 nm) over time (sec). Cells were exposed at the indicated time (arrows) to vehicle media (top), media containing 1000 ng/ml of $PGF_{2\alpha}$ (middle), and media containing 100 nM ET-1 (bottom).

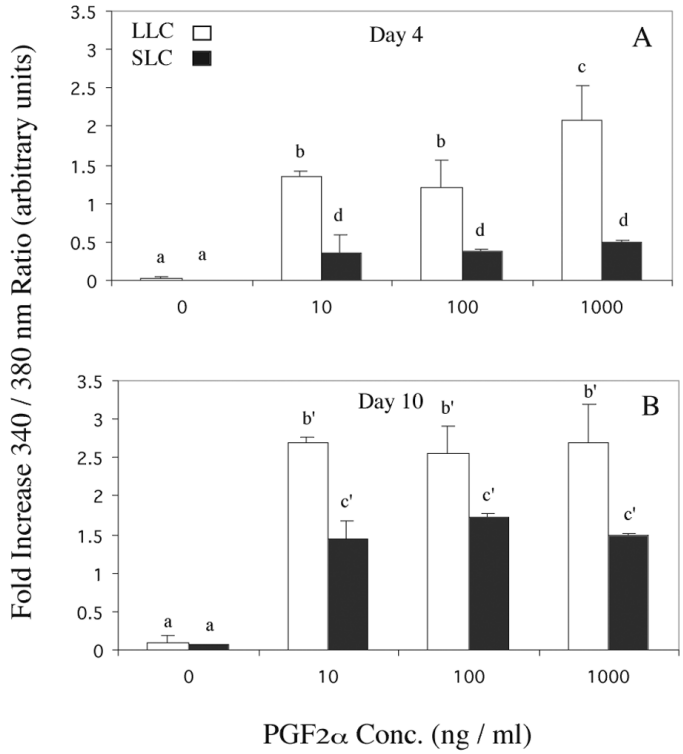

FIG. 3. $PGF_{2\alpha}$-induced changes in the intracellular Ca^{2+} levels in LLC and SLC studied on (**A**) Day 4 (0 ng/ml, n = 121 SLC and 98 LLC; 10 ng/ml, n = 105 SLC and 89 LLC; 100 ng/ml, n = 107 SLC and 118 LLC; 1000 ng/ml, n = 114 SLC and 103 LLC) and (**B**) Day 10 (0 ng/ml, n = 102 SLC and 100 LLC; 10 ng/ml, n = 135 SLC and 126 LLC; 100 ng/ml, n = 125 SLC and 123 LLC; 1000 ng/ml, n = 112 SLC and 133 LLC). Values are presented as mean ± SEM, and comparisons were made within cell type, developmental stage, and treatment. Different letters on top of bars represent significantly different values ($P < 0.05$).

In the SLC population, $PGF_{2\alpha}$ induced elevations in $[Ca^{2+}]_i$ of lower amplitude than those elicited by comparable $PGF_{2\alpha}$ concentration in LLC; furthermore, this amplitude was not increased as the cells were challenged with greater concentrations of $PGF_{2\alpha}$ (Fig. 3A). In SLC, the percentage of responding cells did not increase as concentrations of $PGF_{2\alpha}$ increased ($P = 0.84$); it remained near the 80% ± 5.6% that was observed with the lowest tested concentration (Table 1).

The actions of ET-1 in inducing transient elevations in

TABLE 1. Percentage of SLC and LLC responding to ET-1 and $PGF_{2\alpha}$ with a rise in the intracellular concentration on Ca^{2+} as a function of agonist concentration and luteal development.[a]

	Day 4		Day 10	
	SLC	LLC	SLC	LLC
ET-1 (nM)				
0	1.2 ± 2.3[b] (121)	6.3 ± 4.5[b] (98)	1 ± 1.2[b] (102)	3[b] (100)
10	75 ± 6.3[c] (115)	70 ± 2.3[c] (96)	58.3 ± 2.3[c] (125)	72.7 ± 4.5[c] (123)
100	60 ± 5.5[c] (125)	66 ± 12.1[c] (116)	90 ± 1[d] (104)	80 ± 2.3[c] (114)
1000	80 ± 1.6[c] (110)	57 ± 2.3c (102)	100[d] (103)	81.8 ± 2[c] (116)
$PGF_{2\alpha}$ (ng/ml)				
0	1.2 ± 2.3[b] (121)	6.3 ± 4.5[b] (98)	1 ± 1.2[b] (102)	3[b] (100)
10	80 ± 5.6[c] (105)	100[c] (89)	75 ± 8.1[c] (135)	80 ± 5.6[c] (126)
100	85 ± 5.6[c] (107)	100[c] (118)	71.4 ± 5.6[c] (125)	58 ± 9.8[d] (123)
1000	83 ± 2[c] (114)	88 ± 2.6[c] (103)	100[d] (112)	85.7 ± 4.5[c] (133)

[a] Values represent the mean ± SEM. Numbers in parentheses denote the number of cells in each treatment group. For each cell type, comparisons are made within each agonist concentration.
[b–d] Values marked with different superscripts differ significantly ($P < 0.05$).

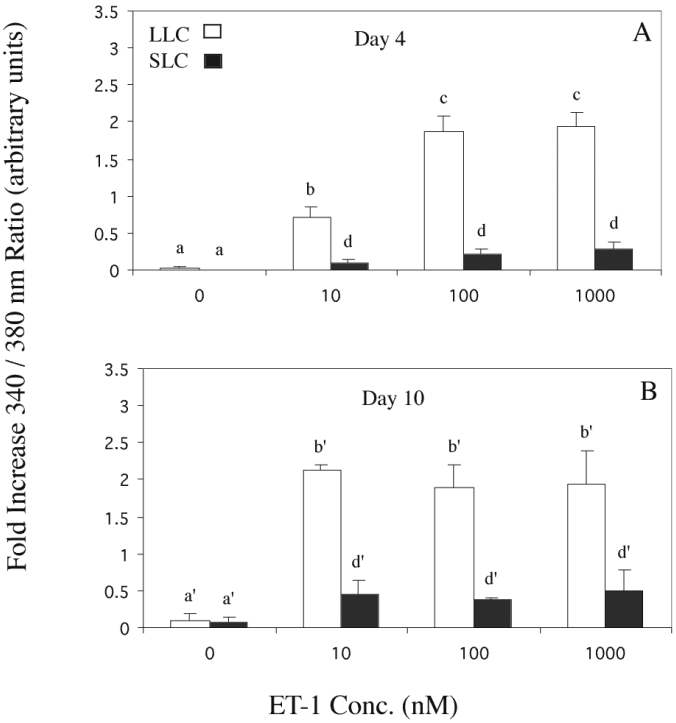

FIG. 4. ET-1-induced changes in the intracellular Ca^{2+} levels in LLC and SLC studied on (**A**) Day 4 (0 nM, n = 121 SLC and 98 LLC; 10 nM, n = 115 SLC and 96 LLC; 100 nM, n = 125 SLC and 116 LLC; 1000 nM, n = 110 SLC and 102 LLC) and (**B**) Day 10 (0 nM, n = 102 SLC and 100 LLC; 10 nM, n = 125 SLC and 123 LLC; 100 nM, n = 104 SLC and 114 LLC; 1000 nM, n = 103 SLC and 116 LLC). Values are presented as the mean ± SEM, and comparisons were made within cell type, developmental stage, and treatment. Different letters on top of bars represent significantly different values ($P < 0.05$).

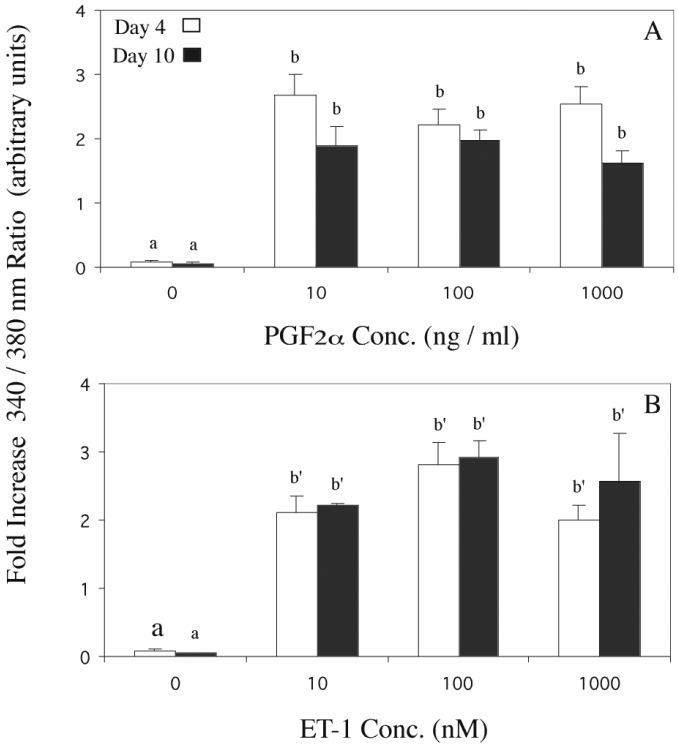

FIG. 5. (**A**) $PGF_{2\alpha}$-induced and (**B**) ET-1-induced changes in the intracellular Ca^{2+} levels in endothelial cells studied on Day 4 ($PGF_{2\alpha}$: 0 ng/ml, n = 126; 10 ng/ml, n = 128; 100 ng/ml, n = 126; 1000 ng/ml, n = 117; ET-1: 0 nM, n = 126; 10 nM, n = 103; 100 nM, n = 117; 1000 nM, n = 120) and on Day 10 ($PGF_{2\alpha}$: 0 ng/ml, n = 124; 10 ng/ml, n = 136; 100 ng/ml, n = 113; 1000 ng/ml, n = 134; ET-1: 0 nM, n = 124; 10 nM, n = 107; 100 nM, n = 128; 1000 nM, n = 128). Values are presented as the mean ± SEM, and comparisons were made within developmental stage and treatment. Letters on top of bars represent significantly different values ($P < 0.05$).

$[Ca^{2+}]_i$ in steroidogenic cells isolated from Day 4 CL were remarkably similar to those described for $PGF_{2\alpha}$. That is, a dose effect of ET-1 on the elicited rise in $[Ca^{2+}]_i$ was observed only in the LLC population. A maximal response in the ET-1-induced transient elevations in $[Ca^{2+}]_i$ was observed at a concentration of 100 nM (Fig. 4A).

As observed for $PGF_{2\alpha}$, the ET-1-induced transient elevations in $[Ca^{2+}]_i$ in the SLC population were of lower amplitude than those elicited by a comparable concentration of ET-1 in LLC. Furthermore, this amplitude was not increased as the cells were challenged with greater concentrations of ET-1 (Fig. 4A). The percentage of SLC responding to a challenge with increasing concentrations of ET-1, as observed for $PGF_{2\alpha}$, did not differ from the percentage that responded to the lowest tested concentration (Table 1).

All concentrations of $PGF_{2\alpha}$ and ET-1 tested on endothelial cells elicited significant and agonist-specific transient elevations in $[Ca^{2+}]_i$. Furthermore, the lowest tested concentration for each agonist elicited maximal responses by endothelial cells (Fig. 5). The percentage of responding endothelial cells was not affected by the concentration of ET-1 used in the challenge. For Day 4 samples, no responders were observed from 126 endothelial cells stimulated with media alone, and when endothelial cells were stimulated with $PGF_{2\alpha}$ (10 ng/ml, n = 128; 100 ng/ml, n = 126; 1000 ng/ml, n = 117), 93% ± 3% of the cells responded with maximal elevations in $[Ca^{2+}]_i$. For Day 10 samples, 1 of 124 endothelial cells stimulated with media responded with an elevation in $[Ca^{2+}]_i$; in contrast, when stimulated with $PGF_{2\alpha}$ (10 ng/ml, n = 136; 100 ng/ml, n = 113; 1000 ng/ml, n = 134), 94–100% of the cells responded with maximal elevations in $[Ca^{2+}]_i$. Similar results were obtained when endothelial cells were stimulated with ET-1. For Day 4 endothelial cells stimulated with ET-1 (10 nM, n = 103; 100 nM; n = 117; 1000 nM, n = 120), 100% of the cells responded with maximal elevations in $[Ca^{2+}]_i$. For Day 10 endothelial cells stimulated with ET-1 (10 nM, n = 107; 100 nM, n = 128; 1000 nM, n = 128), 93% ± 5.6% of the cells responded with maximal elevations in $[Ca^{2+}]_i$.

Day 10 CL. In Day 10 LLC, $PGF_{2\alpha}$ at a concentrations of 10 ng/ml elicited maximal rises in $[Ca^{2+}]_i$. In fact these responses were greater ($P = 0.04$) than those elicited by the highest concentration of $PGF_{2\alpha}$ on Day 4 LLC (Fig. 3), and the percentage of responding cells or the elevations in $[Ca^{2+}]_i$ were not increased as the $PGF_{2\alpha}$ concentration was increased (Fig. 3B and Table 1).

In the SLC population, the lowest concentration of $PGF_{2\alpha}$ induced maximal elevations in $[Ca^{2+}]_i$ that, again, were of lower amplitude than those elicited by comparable concentrations of $PGF_{2\alpha}$ on LLC. Increasing the concentration of $PGF_{2\alpha}$ did not result in a further increase ($P = 0.84$) of the observed responses (Fig. 3B). Nevertheless, the amplitude of these responses was higher ($P < 0.05$) than those elicited by $PGF_{2\alpha}$ in Day 4 SLC (Fig. 3).

Increasing the concentrations of $PGF_{2\alpha}$ resulted in a progressive increase in the percentage of responding SLC. When these SLC were challenged at the concentration of 10 ng/ml, 75% ± 8.1% of the cells responded. It remained at 71.4% ± 5.6% when the $PGF_{2\alpha}$ concentration was increased to 100 ng/ml, and then it was increased to 100%

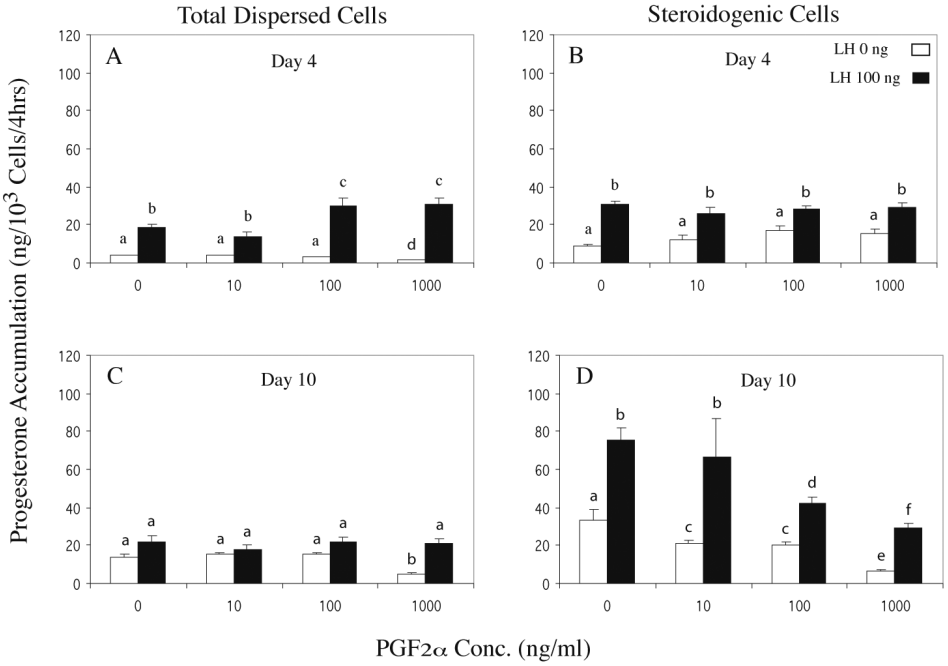

FIG. 6. Effects of $PGF_{2\alpha}$ on progesterone accumulation on Day 4 by (**A**) total dispersed cells and (**B**) steroidogenic cells on Day 10 by (**C**) total dispersed cells and (**D**) and steroidogenic cells. Values are represented as the mean ± SEM. Letters on top of bars represent significantly different values ($P < 0.05$).

when the concentrations of $PGF_{2\alpha}$ was increased to 1000 ng/ml (Table 1).

In LLC, the lowest tested concentration of ET-1 (10 nM) elicited elevations in $[Ca^{2+}]_i$ of maximal amplitude (Fig. 4B). Additionally, the percentage of responding cells was not increased by increasing the ET-1 concentration (Table 1).

As described for $PGF_{2\alpha}$, ET-1 induced lower elevations in $[Ca^{2+}]_i$ in SLC compared with those in LLC ($P = 0.003$) (Fig. 4B). However, these ET-1-stimulated elevations in $[Ca^{2+}]_i$ were greater ($P = 0.002$) in SLC isolated from Day 10 CL than in those isolated from Day 4 CL (Fig. 4). The ET-1-induced Ca^{2+} transients were not affected by the concentration of ET-1 used (Fig. 4B), indicating that they represented a maximal response. The percentage of SLC responding was affected by the concentration of ET-1 used. At the concentration of 10 nM, ET-1 elicited responses in 58.3% ± 2.3% of the cells challenged. This percentage was increased to 90% ± 1% when the concentration of ET-1 was increased to 100 nM, and no additional change was observed when ET-1 was used at the concentration of 1000 nM (Table 1).

The developmental stage of the CL did not influence the sensitivity of the endothelial cells to $PGF_{2\alpha}$ or ET-1; the Ca^{2+} transients elicited by the low agonist concentration were maximal in both developmental stages (Fig. 5). Furthermore, the percentage of responding endothelial cells did not differ with day (data not shown), and the lowest tested concentration of agonist elicited a maximal percentage of responding cells.

Experiment 2: Ability of $PGF_{2\alpha}$ and ET-1 to Inhibit Basal and LH-Stimulated P_4 Production

Day 4 CL. Basal P_4 accumulation in the cultures of total dispersed cells was 3.74 ± 0.33 ng/ml, and a 66% inhibitory effect of $PGF_{2\alpha}$ on the amount of accumulated P_4 was observed only at the highest tested concentration (1000 ng/ml) (Fig. 6A). When these Day 4 total dispersed luteal cells were stimulated with 100 ng/ml of LH, the amount of accumulated P_4 was increased to approximately fivefold that accumulated under basal conditions (Fig. 6A). Superimposing the $PGF_{2\alpha}$ treatment at the concentrations of 100 and 1000 ng/ml significantly enhanced the stimulatory effect of LH ($P = 0.0001$) on the accumulated P_4 (Fig. 6A).

Basal P_4 accumulation in cultures of steroidogenic cells was twice the amount accumulated in the cultures of total dispersed luteal cells. Superimposing the $PGF_{2\alpha}$ treatment on these Day 4 luteal steroidogenic cells did not affect basal or LH-stimulated P_4 accumulation (Fig. 6A).

In the cultures of total dispersed cells used to test the effect of ET-1, basal P_4 accumulation was 3.65 ± 0.38 ng/ml, and ET-1 did not have any effect at any of the concentrations tested (Fig. 7A). When cultures were stimulated with 100 ng/ml of LH, the amount of accumulated P_4 was increased to approximately fivefold that accumulated under basal conditions (Fig. 7A). When ET-1 was included in the treatment, it induced a dose-dependent inhibition of the LH-stimulated P_4 accumulation. At 10 nM, ET-1 significantly inhibited P_4 accumulation by 54.1% (Fig. 7A); increasing the concentration of ET-1 to 100 nM brought about an inhibition of approximately 71.9% (Fig. 7A). Additional increases in the ET-1 concentration did not further increase this inhibitory action on the accumulated P_4 (Fig. 7A).

As in the experiments with $PGF_{2\alpha}$, basal P_4 accumulation in cultures of steroidogenic cells isolated from Day 4 CL was twice the amount accumulated in the cultures of total dispersed luteal cells (Fig. 7B). Superimposing an ET-1 treatment on these steroidogenic cells induced a dose-dependent inhibition of the basal P_4 accumulation (Fig. 7B). The lowest tested concentration of ET-1 (10 nM) did not reduce the amount of P_4 accumulated. However, at 100 nM, ET-1 induced a reduction of approximately 82.9%, and at the concentration of 1000 nM, the inhibition was further increased to approximately 94.2% (Fig. 7B). In contrast, the LH-stimulated P_4 accumulation in these Day 4 steroidogenic cells was significantly and maximally inhibited by the lowest tested concentration of ET-1 (Fig. 7B).

Day 10 CL. Basal P_4 accumulation in cultures of total dispersed cells was approximately 3.6-fold higher than in those isolated from the Day 4 CL, 13.43 ± 5.18 ng/ml. A

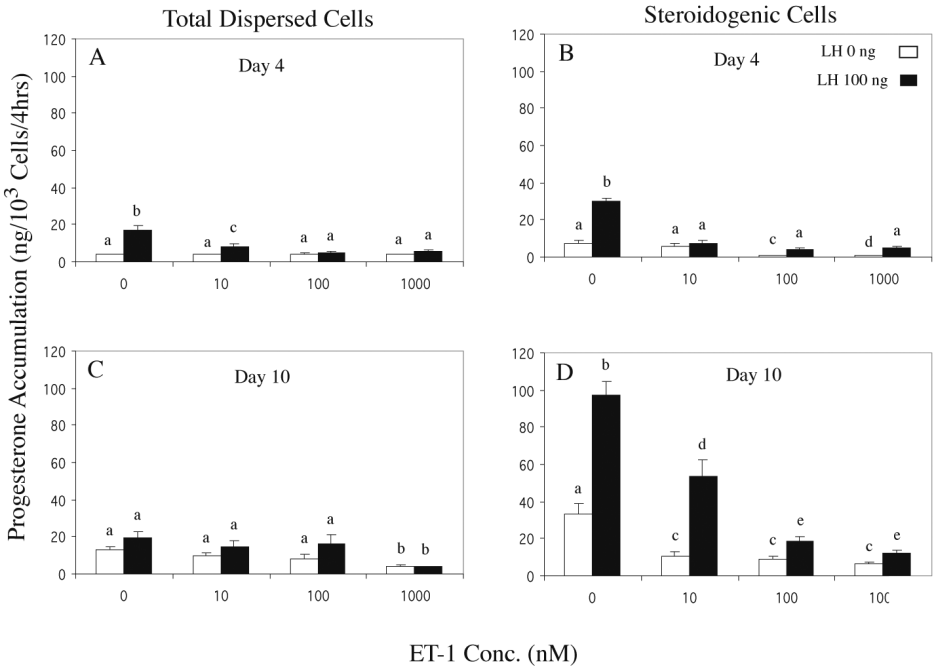

FIG. 7. Effects of ET-1 on progesterone accumulation on Day 4 by (**A**) total dispersed cells and (**B**) steroidogenic cells on Day-10 by (**C**) total dispersed cells and (**D**) steroidogenic cells. Values are presented as the mean ± SEM. Letters on top of bars represent significantly different values ($P < 0.05$).

63% inhibitory effect of $PGF_{2\alpha}$ on the amount of accumulated P_4 was observed only at the highest tested concentration (1000 ng/ml) (Fig. 6C). When Day 10 dispersed luteal cells were stimulated with 100 ng/ml of LH, the amount of accumulated P_4 was increased to approximately 1.6-fold that accumulated under basal conditions (Fig. 5B). Superimposing a $PGF_{2\alpha}$ treatment on these luteal steroidogenic cells did not affect their LH-stimulated P_4 accumulation (Fig. 6C).

Again, basal P_4 accumulation in steroidogenic cell cultures was more than double the amount accumulated in cultures where endothelial cells were present. Superimposing a $PGF_{2\alpha}$ treatment on these cells induced a dose-dependent inhibition of basal P_4 accumulation (Fig. 6D). The lowest tested concentration of $PGF_{2\alpha}$ (10 ng/ml) induced approximately 37% inhibition, and at 1000 ng/ml, $PGF_{2\alpha}$ induced an inhibition of approximately 80% (Fig. 6D). When cultures were stimulated with 100 ng/ml of LH, the amount of accumulated P_4 was increased to approximately 2.25-fold that accumulated under basal conditions (Fig. 6B). Superimposing the $PGF_{2\alpha}$ treatment on these steroidogenic cells induced a dose-dependent inhibition of the LH-stimulated P_4 accumulation (Fig. 6D). The lowest tested concentration of $PGF_{2\alpha}$ (10 ng/ml) did not have any effect, but at a concentration of 100 ng/ml, an inhibition of approximately 37% was observed (Fig. 6D). At the concentration of 1000 ng/ml, an inhibition of approximately 61.5% was induced (Fig. 6D).

Again, in cultures of total dispersed luteal cells used to examine the effect of ET-1, basal P_4 accumulation on Day 10 was higher than that in those isolated from the Day 4 CL (Fig. 7C). An ET-1-induced inhibitory effect on the amount of accumulated P_4 was observed only at the highest tested concentration (1000 nM) (Fig. 7C). When these cells were stimulated with 100 ng/ml of LH, the amount of accumulated P_4 was increased to approximately 1.6-fold above that accumulated under basal conditions (Fig. 7C). An ET-1-induced inhibitory effect was observed only at the highest tested concentration (1000 nM) (Fig. 7C).

In contrast, in steroidogenic cells, ET-1, even at 10 nM, maximally inhibited basal P_4 accumulation. The maximal inhibitory effect of ET-1 on basal P_4 accumulation was 79.7% (Fig. 7D). Luteinizing hormone stimulated P_4 accumulation to 97.10 ± 7.12 ng/ml, a 2.9-fold increase over basal accumulation, and ET-1, in a dose-dependent manner, inhibited this stimulatory effect of LH. At 10 nM, ET-1 induced an inhibition of approximately 45% (53.2 ± 8.78 ng/ml), and at 100 nM, the inhibition was further augmented to approximately 81% (18.28 ± 2.27 ng/ml). The highest tested concentration of ET-1 (1000 nM) further decreased the LH-induced P_4 accumulated by 87% (Fig. 7D).

DISCUSSION

The results of experiment 1 demonstrate developmental differences in the ability of $PGF_{2\alpha}$ to evoke increases in $[Ca^{2+}]_i$ in both steroidogenic cell types of the bovine CL. The LLC population of the Day 10 CL had greater sensitivity to $PGF_{2\alpha}$ than that of the Day 4 CL. This interpretation is supported by the observation made in Day 10 LLC that the lowest concentration of $PGF_{2\alpha}$ elicited maximal responses in terms of amplitude of the calcium rise and in terms of the percentage of responding cells. A greater sensitivity of the Day 10 CL to $PGF_{2\alpha}$ also was supported by two observations made in the SLC population. First, $PGF_{2\alpha}$ was able to evoke increases in $[Ca^{2+}]_i$ in Day 10 SLC that were of greater amplitude than those elicited in Day 4 SLC. Second, in Day 10 SLC, the percentage of responding cells increased as a function of the $PGF_{2\alpha}$ concentration used. Consequently, these developmental differences in the steroidogenic cells could result in an increased ability of $PGF_{2\alpha}$ to evoke greater increases in $[Ca^{2+}]_i$ in both steroidogenic cell types of the Day 10 bovine CL. These functional findings are consistent with those of previous studies showing that both small and large bovine luteal cells express $PGF_{2\alpha}$ receptors [2, 20, 29]. Nevertheless, to our knowledge, the present study is the first to demonstrate the ability of $PGF_{2\alpha}$ to evoke increases in $[Ca^{2+}]_i$ in SLC and to document developmental differences in this ability of $PGF_{2\alpha}$ to evoke increases in $[Ca^{2+}]_i$ in bovine luteal steroidogenic cells. Differences in the magnitude and profiles of agonist-stimulated increases in $[Ca^{2+}]_i$ in SLC and LLC have been

previously and widely reported in bovine and ovine luteal cells [29–35]. Interestingly, Alila et al. [34] reported differences in the LH-stimulated increases in $[Ca^{2+}]_i$ in SLC and LLC of the bovine CL, but for LH, it was SLC that had higher-amplitude responses compared with LLC.

The present results, although stressing the importance of the intracellular effectors associated with the $PGF_{2\alpha}$ receptor, do not allow an explanation of the cellular mechanism responsible for this developmental difference. The in vivo insensitivity to $PGF_{2\alpha}$ in the early CL is not attributable to a deficiency of high-affinity $PGF_{2\alpha}$ receptors [18]. Nevertheless, it is not known if changes in receptor concentrations in specific cell types could explain the developmental differences documented in the present study. Alternatively, it may not be the number of $PGF_{2\alpha}$ receptors that is increased significantly with the development of the CL but, rather, some aspect of the signal transduction mechanism associated with the receptor changes. As demonstrated in experiment 1, the $PGF_{2\alpha}$ receptor is still coupled to the free calcium intracellular mediator in both developmental stages, but $PGF_{2\alpha}$ action on luteal cells of the early phase CL is less effective in eliciting a calcium signal. This could be caused by the lack of one component of the signal transduction pathway that is present during a later developmental stage. Indeed, we have recently reported that the early CL expresses lower amount of PKC ε than the midphase CL [36]. Various PKC isozymes are themselves involved in regulating agonist-induced Ca^{2+} signaling in different cell types; for example, PKC ε is necessary for initiation of LTD4-induced Ca^{2+} signaling in intestinal epithelial cells [37]. A recent study by Sen et al. (unpublished results), in which a PKC isozyme-specific inhibitor was used to block PKC ε selectively, indicates that this isozyme is capable of modulating the Ca^{2+} signaling ability of $PGF_{2\alpha}$. It could be that expression of the full array of PKC isozymes during the midluteal phase confers a broader network of intracellular mediators, transducing a full range of luteolytic actions of $PGF_{2\alpha}$ in the Day 10 CL. Partial expression of the array of PKC isozymes at earlier developmental stages would render the tissue differentially sensitive to alternative effects of $PGF_{2\alpha}$ (i.e., stimulatory vs. inhibitory actions). Nevertheless, important to the validity of generalizing these possible interpretations, clear evolutionary species differences exist in this regard, because in the sheep and pig, $PGF_{2\alpha}$ functions and receptors have been shown to localize primarily in the LLC [30–32, 38–40].

The results of experiment 1 demonstrate developmental differences in the ability of ET-1 to evoke increases in $[Ca^{2+}]_i$ in both steroidogenic cell types of the bovine CL. The LLC and SLC populations of the Day 10 CL had slightly greater sensitivity to ET-1 than those of the Day 4 CL. This is documented clearly by the observation that in both cell populations of the Day 10 CL, the lowest tested concentration of ET-1 elicited maximal responses in terms of amplitude of the calcium rise. This ability of ET-1 to evoke increases in $[Ca^{2+}]_i$ of rapid kinetics in both steroidogenic cell types of the bovine CL is consistent with the interpretation that the ET receptors expressed in the bovine CL are linked to the PLC effector system. This finding is in accord with previous reports that the ET receptors expressed in porcine granulosa cells were characterized by their ability to mediate metabolism of membrane inositol phospholipids, evoking increases in $[Ca^{2+}]_i$ and activating PKC [25, 26].

In contrast to the developmental differences in the steroidogenic luteal cell populations, the sensitivity of the luteal endothelial cell populations to $PGF_{2\alpha}$ and ET-1 does not appear to contribute to the luteolytic insensitivity of the early CL. Both agonists were able to elicit maximal responses at the lowest concentration tested, regardless of the developmental stage of the CL. Therefore, at least regarding $[Ca^{2+}]_i$ (a likely relevant intracellular mediator), this observation does not support the suggestion that endothelial cells of the early CL are not ready developmentally to mediate the luteolytic action of $PGF_{2\alpha}$ [6].

The results of experiment 2 agree with the developmental differences observed in the ability of $PGF_{2\alpha}$ to evoke increases in $[Ca^{2+}]_i$ in the steroidogenic luteal cells and stresses the importance of $[Ca^{2+}]_i$ in mediating the luteolytic actions of $PGF_{2\alpha}$. The effectiveness of $PGF_{2\alpha}$ in inhibiting basal and LH-stimulated P_4 accumulation was observed only in cultures of steroidogenic cells isolated from Day 10 CL. These findings contrast with the idea proposed in a previous report [3] that $PGF_{2\alpha}$ does not directly inhibit P_4 production in luteal steroidogenic cells if the endothelial cells are absent from the tissue culture. However, the experimental observations for this proposition were based on experiments conducted with ''luteal-like'' cells (''LLC'' and ''SLC'') and luteal slices. These ''LLC'' and ''SLC'' were collected from follicles and then luteinized in vitro. It is possible that these ''luteal-like'' cells resemble luteal cells from the early phase rather than cells from the midphase CL. This technical difference could explain the discrepancy between these two studies; in the present study, a direct effect of $PGF_{2\alpha}$ in inhibiting basal and LH-stimulated P_4 accumulation was observed only in steroidogenic cell cultures from Day 10 CL.

The relationship between the inhibitory effectiveness of $PGF_{2\alpha}$ and the two developmental stages was difficult to ascertain in cell cultures of total dispersed luteal cells. The more complicated cellular interactions under these conditions are reflected by the observation that P_4 accumulation consistently was suppressed when the endothelial cells were cocultured with the steroidogenic cells. Interestingly, this was observed at both developmental stages. This suppression in P_4 accumulation by endothelial cells most likely was caused, though not entirely, by the ET-1 secreted by the endothelial cells. Our preliminary evidence indicates that this suppression is blocked only partially by a combination of type A and B ET-receptor antagonists (unpublished observations), indicating the presence of additional factors besides ET-1 under these tissue-culture conditions. Nevertheless, it is of interest that under these conditions in the Day 4 dispersed luteal cells, $PGF_{2\alpha}$ enhanced the stimulatory effect of LH at concentrations of 100 and 1000 ng/ml, but inhibited basal P_4 accumulation only at the highest concentration, in both Day 4 and Day 10 luteal cells. This stimulatory action of PGs has been observed in previous studies [41], but in the present study, the stimulatory action of $PGF_{2\alpha}$ was observed in the Day 4 CL only when the endothelial cells were present. These results raise the possibility that at this developmental stage, the CL not only is less sensitive to $PGF_{2\alpha}$ but also that the nature of the effects of $PGF_{2\alpha}$ on P_4 accumulation is different from that of those effects observed later during luteal development. As mentioned, the cellular explanation for this observation could be that partial expression of the array of PKC isozymes at earlier developmental stages renders the tissue of the early CL differentially sensitive to alternative effects of $PGF_{2\alpha}$.

The results of experiment 2 demonstrate that ET-1, unlike $PGF_{2\alpha}$, is a paracrine luteolytic agent that has the capacity to reduce both basal and LH-stimulated P_4 accu-

mulation in the Day 4 and Day 10 bovine CL with similar potency. Taken together, the comparison of the developmental sensitivity of the bovine CL to $PGF_{2\alpha}$ and ET-1 indicates that the role of ET-1 cannot be simply to mediate the $PGF_{2\alpha}$-induced decrease in P_4 secretion during luteal regression. Instead, the relationship between these two important luteolytic agents at this stage of luteal development is rather additive. Endothelin-1 and $PGF_{2\alpha}$, each independently of the other, inhibits P_4 production by steroidogenic luteal cells. This interpretation is supported by the report that an intraluteal injection of an ET-receptor antagonist before i.m. injection of 10 mg of PG agonist only mitigated the luteolytic effect of $PGF_{2\alpha}$ [13]. A synergistic interaction between $PGF_{2\alpha}$ and ET-1 on the release of P_4 has been observed in the ovine CL [42]. Two important corollaries are derived from this minor, but also important, interpretation about the relationship between these two naturally occurring luteolytic agents. First, the insensitivity of the early CL to the ability of $PGF_{2\alpha}$ to induce a decrease in P_4 secretion is related more to the differences in developmental sensitivity to $PGF_{2\alpha}$ than to the absence of ET-1 actions to mediate the luteolytic action of $PGF_{2\alpha}$. In late-phase CL, tonic inhibition by ET-1 would be additive with $PGF_{2\alpha}$ and, possibly, with several other well-documented changes that occur at this developmental stage of the CL. For example, catabolism of $PGF_{2\alpha}$ in the late-phase CL is lower than that in the $PGF_{2\alpha}$-resistant CL [14], and $PGF_{2\alpha}$ induces expression of PGG/PGH synthetase-2 in the ovine CL during luteolysis, establishing a potential positive feedback at this stage [15]. Also, local interaction of $PGF_{2\alpha}$ with ET-1 and tumor necrosis factor α on the release of P_4 and oxytocin in ovine CLs in vivo has been reported [42]. Consequently, it is the additive effect of these developmental changes that would increase, or enhance, the luteolytic action of $PGF_{2\alpha}$. Second, the fact that the two developmental stages examined have similar sensitivity to ET-1 begs the question as to the role of ET-1 during the early phase of the CL. As shown by the results of experiment 2, when endothelial cells were present in the dispersed luteal cells from Day 4 and Day 10 CL, P_4 accumulation was similarly suppressed. This indicates that the role of ET-1 in the early and late CL is the same: tonic inhibition of P_4 secretion. Although exogenous $PGF_{2\alpha}$ does not induce ET-1 synthesis, evidence exists for up-regulation in the gene expression encoding the type A ET receptor and biologically active ET-1 peptide over time that is independent of exogenous $PGF_{2\alpha}$ in the early bovine CL [17, 43]. Consequently, the amounts of ET-1 present in the Day 4 and Day 10 CL were not different [17]. The physiological significance of maintaining tonic inhibition of P_4 synthesis might be related to pulsatile secretion of P_4 to prevent P_4 desensitization of the target tissues. Most hormones are secreted in an episodic manner, and secretion of P_4 has been shown to be episodic in nature [44].

The observation that ET-1 is able to stimulate a rise in $[Ca^{2+}]_i$ in both SLC and LLC is in accord with the reports that ET-1 receptors are found in both cell types. However, ET-1 failed to inhibit P_4 production by in vitro-luteinized bovine thecal cells (SLC-like cells) [5]. The data from the present study do not allow us to distinguish if the calcium signal translates into an inhibitory action of ET-1 in both or only one steroidogenic cell. However, if the in vitro-luteinized SLC are like natural SLC, then there could be an implication that in SCL, the ET-1-stimulated calcium signal could be associated with a different cellular response than inhibiting P_4 synthesis.

In summary, the potency of ET-1 to inhibit P_4 synthesis in the Day 4 and Day 10 CL was not different; therefore, the luteal role for ET-1 appears to be more of a tonic inhibitor of P_4 synthesis than a mediator of $PGF_{2\alpha}$ action. Because in the early CL the ET system is up-regulated over time in a manner that is independent of exogenous $PGF_{2\alpha}$ and ET-1 is capable of inhibiting P_4 synthesis, it is unlikely at this developmental stage that the inability of $PGF_{2\alpha}$ to up-regulate the ET-1 could account for the insensitivity of the early CL. Instead, the lower efficacy of $PGF_{2\alpha}$ in decreasing P_4 secretion in the early CL more likely might relate to developmental differences in the signal transduction associated with the $PGF_{2\alpha}$ receptor at these two developmental stages.

REFERENCES

1. O'Shea JD, Rodgers RJ, D'Occhio MJ. Cellular composition of cyclic corpus luteum of the cow. J Reprod Fertil 1989; 85:483–487.
2. Mamluk R, Chen D, Greber Y, Davis JS, Meidan R. Characterization of prostaglandin $F_{2\alpha}$- and LH-receptor mRNA expression in different bovine luteal cell types. Biol Reprod 1998; 58:849–856.
3. Girsh E, Milvae RA, Wang W, Meidan R. Effect of endothelin-1 on bovine luteal function: role in prostaglandin $PGF_{2\alpha}$-induced antisteroidogenic action. Endocrinology 1996; 137:1306–1312.
4. Mamluk R, Greber Y, Meidan R. Hormonal regulation of the messenger ribonucleic acid expression of steroidogenic factor-1, steroidogenic acute regulatory protein, and cytochrome P450 side-chain cleavage in bovine luteal cells. Biol Reprod 1999; 60:628–634.
5. Girsh E, Greber Y, Meidan R. Luteotrophic and luteolytic interactions between bovine small and large luteal-like cells and endothelial cells. Biol Reprod 1995; 52:954–962.
6. Levy N, Kobayashi S, Roth Z, Wolfenson D, Miyamoto A, Meidan R. Administration of prostaglandin $F_{2\alpha}$ during the early bovine luteal phase does not alter the expression of ET-1 and of its type A receptor: a possible cause for corpus luteum refractoriness. Biol Reprod 2000; 63:377–382.
7. Meidan R, Milvae RA, Weiss S, Levy N, Friedman A. Intra-ovarian regulation of luteolysis. J Reprod Fertil Suppl 1999; 54(suppl):217–228.
8. Girsh E, Wank A, Mamluk A, Arditi F, Friedman A, Milvae RA. Regulation of endothelin-1 expression in the bovine corpus luteum: elevation by prostaglandin $F_{2\alpha}$. Endocrinology 1996; 137:5191–5196.
9. Ohtani M, Kobayashi S, Miyamoto A, Hayashi K, Fukui Y. Real-time relationships between intraluteal and plasma concentration of endothelin, oxytocin, and progesterone during prostaglandin $PGF_{2\alpha}$-induced luteolysis in the cow. Biol Reprod 1998; 58:103–108.
10. Milvae RA. Interrelationships between endothelin and prostaglandin $PGF_{2\alpha}$ in the corpus luteum function. Rev Reprod 2000; 5:1–5.
11. Wright MF, Sayre B, Inskeep EK, Flores JA. Prostaglandin $F_{2\alpha}$ regulation of the bovine corpus luteum endothelin system during the early and mid luteal phase. Biol Reprod 2001; 65:1710–1717.
12. Levy N, Gordin M, Mamluk R, Yanagisawa M, Smith M, Hampton JH, Meidan R. Distinct cellular localization and regulation of endothelin-1 and endothelin converting enzyme-1 expression in the bovine corpus luteum: implications for luteolysis. Endocrinology 2001; 142: 5254–5260.
13. Hinckley ST, Milvae RA. Endothelin-1 mediates prostaglandin $F_{2\alpha}$-induced luteal regression in the ewe. Biol Reprod 2001; 64:1619–1623.
14. Silva PJ, Juengel JL, Rollynson MK, Niswender GD. Prostaglandin metabolism in the ovine corpus luteum: catabolism of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) coincides with resistance of the corpus luteum to $PGF_{2\alpha}$. Biol Reprod 2000; 63:1229–1236.
15. Tsai SJ, Wiltbank MC. Prostaglandin $F_{2\alpha}$ induces expression of prostaglandin G/H synthetase-2 in the ovine corpus luteum: a potential positive feedback loop during luteolysis. Biol Reprod 1997; 57:1016–1022.
16. Sayre BL, Taft R, Inskeep EK, Killefer J. Increased expression of insulin-like growth factor-binding protein-1 during induced regression of bovine corpora lutea. Biol Reprod 2000; 63:21–29.
17. Choudhary E, Costine BA, Wilson ME, Inskeep EK, Flores JA. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) independent and dependent regulation of the bovine luteal endothelin system. Domest Anim Endocrinol 2004; 27: 63–79.

18. Wiltbank MC, Shio TF, Bergfelt DR, Ginther OJ. Prostaglandin $F_{2\alpha}$ receptors in the early bovine corpus luteum. Biol Reprod 1995; 52: 74–78.
19. Davis JS, Weakland LL, Weiland DA, Farese RV, West LA. Prostaglandin $F_{2\alpha}$ stimulates phosphatidylinositol 4,5-biphosphate hydrolysis and mobilizes intracellular Ca^{2+} in bovine luteal cells. Proc Natl Acad Sci U S A 1987; 84:3728–3732.
20. Davis JS, Alila HW, West LA, Corradino RA, Hansel W. Acute effects of prostaglandin $F_{2\alpha}$ on inositol phospholipid hydrolysis in the large and small cells of bovine corpus luteum. Mol Cell Endocrinol 1988; 58:43–50.
21. Wiltbank MC, Diskin MG, Niswender GD. Differential actions of second messenger system in the corpus luteum. J Reprod Fertil Suppl 1991; 43:65–75.
22. Casida LE. Research techniques in physiology of reproduction in the female. In: Chapman AB (ed.), Techniques and Procedures in Animal Production Research. Albany, NY: American Society of Animal Production; 1959:106–121.
23. Flores JA, Veldhuis JD, Leong DA. Follicle-stimulating hormone evokes an increase in intracellular free calcium ion concentrations in single ovarian (granulosa) cells. Endocrinology 1990; 127:3172–3179.
24. Flores JA, Veldhuis JD, Leong DA. Angiotensin II induces calcium release in a subpopulation of single ovarian (granulosa) cells. Mol Cell Endocrinol 1991; 81:1–10.
25. Flores JA, Quyyumi S, Leong DA, Veldhuis JD. Actions of endothelin-1 on swine ovarian (granulosa cells). Endocrinology 1992; 131: 1350–1358.
26. Flores JA, Garmey JC, Lahav M, Veldhuis JD. Mechanisms underlying endothelin's inhibition of FSH-stimulated progesterone production by ovarian granulosa cells. Mol Cell Endocrinol 1999; 156:169–178.
27. Sheffel CE, Pratt BR, Ferrell WL, Inskeep EK. Induced corpora lutea in the postpartum beef cow. II. Effects of treatment with progestogen and gonadotropins. J Anim Sci 1982; 54:830–836.
28. Statistical Software for the Apple Macintosh. JMP Statistics and Graphics Guide, Version 3.0. Cary, NC: Statistical Analysis System; 1994.
29. Alila HW, Dowd JP, Corradino RA, Harris WV, Hansel W. Control of progesterone production in small and large bovine luteal cells separated by flow cytometry. J Reprod Fertil 1988; 82:645–655.
30. Wegner JA, Martinez-Zaguilan R, Wise ME, Gillies RJ, Hoyer PB. Prostaglandin $F_{2\alpha}$-induced calcium transient in ovine large luteal cells: I. Alterations in cytosolic-free calcium levels and calcium flux. Endocrinology 1990; 127:3029–3037.
31. Martinez-Zaguilan R, Wegner JA, Gillies RJ, Hoyer PB. Differential regulation of Ca^{2+} homeostasis in ovine large and small luteal cells. Endocrinology 1994; 135:2099–2108.
32. Wegner JA, Martinez-Zaguilan R, Gillies RJ, Hoyer PB. Prostaglandin $F_{2\alpha}$-induced calcium transient in ovine large luteal cells: II. Modulation of the transient and resting cytosolic free calcium alters progesterone secretion. Endocrinology 1991; 128:929–936.
33. Wiltbank MC, Guthrie PB, Mattson MP, Kater SB, Niswender GD. Hormonal regulation of free intracellular calcium concentrations in small and large ovine luteal cells. Biol Reprod 1989; 41:771–778.
34. Alila HW, Corradino RA, Hansel W. Differential effects of luteinizing hormone on intracellular free Ca^{2+} in small and large bovine luteal cells. Endocrinology 1989; 124:2314–2320.
35. Alila HW, Davis JS, Dowd JP, Corradino RA, Hansel W. Differential effects of calcium on progesterone production in small and large bovine luteal cells. J Steroid Biochem 1990; 36:687–693.
36. Sen A, Browning J, Inskeep EK, Lewis P, Flores JA. Expression and activation of protein kinase C isozymes by prostaglandin $F_{2\alpha}$ in the early and midluteal phase bovine corpus luteum. Biol Reprod 2004; 70:379–384.
37. Thodeti CK, Nielsen CK, Paruchuri S, Larsson C, Sjolander A. The epsilon isoform of PKC is involved in regulation of the LTD_4-induced calcium signal in human intestinal epithelial cells. Exp Cell Res 2001; 262:95–103.
38. Niswender GD, Nett TM. Corpus luteum and its control in infraprimate species. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, vol. 1, 2nd ed. New York: Raven Press; 1994:781–816.
39. Yuan W, Connor ML. Protein kinase C activity and its effect on progesterone production by large and small porcine luteal cells. Proc Soc Biol Med 1997; 216:86–92.
40. Mamluk R, Levy N, Rueda B, Davis JS, Meidan R. Characterization and regulation of type A endothelin receptor gene expression in bovine luteal cell types. Endocrinology 1999; 140:2110–2116.
41. Alila HW, Corradino RA, Hansel W. A comparison of the effects of cyclooxygenase prostanoids on progesterone production by small and large bovine luteal cells. Prostaglandins 1988; 36:259–270.
42. Ohtani M, Takase S, Wijayagunawardane MPB, Tetsuka M, Miyamoto A. Local interaction of prostaglandin $F_{2\alpha}$ with endothelin-1 and tumor necrosis factor-α on the release of progesterone and oxytocin in ovine corpora lutea in vivo: a possible implication for a luteolytic cascade. Reproduction 2004; 127:117–124.
43. Berisha B, Schams D, Miyamoto A. The expression of angiotensin and endothelin system members in bovine corpus luteum during estrous cycle and pregnancy. Endocrine 2002; 19:305–312.
44. Filicori M, Butler JP, Crowley WF Jr. Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion. J Clin Invest 1984; 73:1638–1647.