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Effects of Selective Protein Kinase C Isozymes in Prostaglandin$_{2\alpha}$-Induced Ca^{2+} Signaling and Luteinizing Hormone-Induced Progesterone Accumulation in the Mid-Phase Bovine Corpus Luteum[1]

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ABSTRACT

A single-cell approach for measuring the concentration of cytoplasmic calcium ions ($[Ca^{2+}]_i$) and a protein kinase C-epsilon (PKCϵ)-specific inhibitor were used to investigate the developmental role of PKCϵ in the prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$)-induced rise in $[Ca^{2+}]_i$ and the induced decline in progesterone accumulation in cultures of cells isolated from the bovine corpus luteum. $PGF_{2\alpha}$ increased $[Ca^{2+}]_i$ in Day 4 large luteal cells (LLCs), but the response was significantly lower than in Day 10 LLCs (4.3 ± 0.6, n = 116 vs. 21.3 ± 2.3, n = 110). Similarly, the fold increase in the $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ in Day 4 small luteal cells (SLCs) was lower than in Day 10 SLCs (1.6 ± 0.2, n = 198 vs. 2.7 ± 0.1, n = 95). A PKCϵ inhibitor reduced the $PGF_{2\alpha}$-elicited calcium responses in both Day 10 LLCs and SLCs to 3.5 ± 0.3 (n = 217) and 1.3 ± 0.1 (n = 205), respectively. $PGF_{2\alpha}$ inhibited LH-stimulated progesterone (P_4) accumulation only in the incubation medium of Day 10 luteal cells. Both conventional and PKCϵ-specific inhibitors reversed the ability of $PGF_{2\alpha}$ to decrease LH-stimulated P_4 accumulation, and the PKCϵ inhibitor was more effective at this than the conventional PKC inhibitor. In conclusion, the evidence indicates that PKCϵ, an isozyme expressed in corpora lutea with acquired $PGF_{2\alpha}$ luteolytic capacity, has a regulatory role in the $PGF_{2\alpha}$-induced Ca^{2+} signaling in luteal steroidogenic cells, and that this in turn may have consequences (at least in part) on the ability of $PGF_{2\alpha}$ to inhibit LH-stimulated P_4 synthesis at this developmental stage.

calcium, corpus luteum, ovary, progesterone, signal transduction

INTRODUCTION

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is generally recognized as the major luteolytic factor in domestic ruminants [1]. In bovine and ovine corpora lutea, the luteolytic action of $PGF_{2\alpha}$ has been shown to be mediated by G-protein-coupled $PGF_{2\alpha}$ receptors that activate the phospholipase C (PLC) effector system [2]. Indeed, stimulation of luteal cells with $PGF_{2\alpha}$ leads to the rapid accumulation of inositol 1,4,5-trisphosphate (i.p.$_3$) and 1,2-diacylglycerol (DAG). When it binds to its receptor in the endoplasmic reticulum, i.p.$_3$ stimulates an increase in the concentration of cytoplasmic calcium ions ($[Ca^{2+}]_i$) [2–4]. The increases in $[Ca^{2+}]_i$ and DAG result in activation of another intracellular mediator of hormone actions, protein kinase C (PKC). Thus, PKC and calcium are two major intracellular mediators of the luteolytic actions of $PGF_{2\alpha}$ [2, 5].

PKC is a family of serine-threonine kinases that exist as at least 11 closely related isozymes. They are classified into four categories: conventional (designated as α, βI, βII, and γ), novel (δ, α, ψ, ϵ, ϑ, and η), atypical (ζ and λ), and PKCμ. The cofactors required for activation of the isozymes differ in each category. Conventional isozymes are calcium-dependent, whereas novel isoforms are not. However, both conventional and novel isozymes require DAG and phosphatidyl-serine for their activation [6–8]. Moreover, the atypical PKC isozymes are calcium- and DAG-independent [6–8]. A hallmark of PKC activation is its redistribution from one cytoplasmic compartment to another [9]. Translocation of PKCs is mediated by isozyme-specific anchoring proteins termed RACKs [10, 11]. RACK binding results in anchoring the activated PKC isozyme near its substrate. Phosphorylation of the PKC substrate then leads to isozyme-specific physiological responses. Thus, the subcellular localization and functional specificity of activated PKC isozymes depend on their binding to their corresponding RACK [6, 8, 12, 13].

In addition to the Ca^{2+} requirement for activation of conventional PKC isozymes, various isozymes are themselves involved in regulating agonist-induced Ca^{2+} signaling in different cell types [14–21]. For example, PKC-epsilon (PKCϵ) is necessary for initiation of leukotriene D_4-induced Ca^{2+} signaling in intestinal epithelial cells [22]. And in neurons, PKCϵ regulates Ca^{2+} signaling by modulating N-type Ca^{2+} channels [23]. Additional actions of PKC on Ca^{2+} signaling include reducing intracellular calcium storage capacity and augmenting Ca^{2+} entry with submaximal intracellular calcium pool depletion [24], mediation of a negative feedback loop involved in inhibition of i.p.$_3$ production with a consequent constant frequency of $[Ca^{2+}]_i$ oscillations in mouse lachrymal acinar cells [25], PKC-stimulated modulation of i.p.$_3$/Ca^{2+} signaling in the submandibular duct cell line A253 [26], and PKC activation of capacitative calcium entry in an insulin-secreting cell line RINm5F [27].

Sensitivity of the corpus luteum (CL) to luteolytic actions of $PGF_{2\alpha}$ is affected by luteal development. The CL of the early estrous cycle (Days 1–5) is resistant to the luteolytic action of a dose of $PGF2_\alpha$ that induces luteolysis in the mid to late CL (Days 8–15). The mechanisms responsible for this insensitivity are not fully understood but it is likely that several mechanisms are integrated in diverse populations of luteal cells. A recent study [28] demonstrat-

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ed that the array of PKC isozymes expressed in the bovine CL includes α, βI, βII, ϵ, and μ. Moreover, PKCϵ was differentially expressed according to the developmental stage of the CL [28]. Based on these observations, we proposed that differential expression of PKCϵ could contribute to the cellular mechanisms responsible for the relative insensitivity of the early CL to the luteolytic actions of $PGF_{2\alpha}$ [28]. However, specific roles of specific PKC isozymes in CL physiology have received little attention to date.

In this study, a PKCϵ-specific inhibitor peptide (ϵV1–2) was used to investigate the role of this isozyme in previously well-characterized luteolytic actions of $PGF_{2\alpha}$. This inhibitor is 6–8 amino acids long and is derived from the RACK-binding site for PKCϵ. Binding of the inhibitor peptide to PKCϵ-specific RACK blocks binding of the activated isozyme to RACK, thereby preventing its translocation and its physiological response [29–31]. Effective delivery of inhibitor into cells is achieved by means of peptide-delivery technology [32–34]. Once in the cell, the bond linking the inhibitor to a carrier peptide is cleaved, releasing the free inhibitory peptide. The use of this technology in combination with saponin-mediated cell permeabilization facilitates cellular entry of inhibitory peptides [32–34]. The specificity of these isozyme-selective peptides has been fully characterized in a variety of cell types, including intestinal epithelial cells [35], pancreatic cells [36], esophageal smooth cells [37], and vascular smooth muscle cells [38].

The aim of this study was to use a PKCϵ-specific inhibitor to investigate the role of this isozyme in two luteal end points: 1) a $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$, and 2) a $PGF_{2\alpha}$-induced decline in basal and LH-induced progesterone accumulation. The potential role of PKCϵ in these aspects of luteal physiology was investigated during the early luteal and midluteal phases, developmental stages at which PKCϵ is known to be expressed at low and high concentrations, respectively. Based on the results, it is suggested that PKCϵ plays an important role in the ability of $PGF_{2\alpha}$ to induce these two effects on the midphase bovine CL.

MATERIALS AND METHODS

Tissue Collection

Nonlactating 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 [39]. After two cycles, four Day-4 and seven Day-10 corpora lutea were collected by ovariectomy (Day 4) or blunt dissection (Day 10) via supravaginal incision under epidural anesthesia. For the epidural anesthesia, 6–9 ml of 2% lidocaine were administered for 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. The West Virginia University Animal Care and Use Committee reviewed and approved the protocol for the tissue collection (Animal Care and Use Committee 01-0809).

Luteal Cell Dispersion and Purification

In the laboratory, the corpora lutea were dissected free of connective tissue, weighed, placed in cell-dispersion medium (CDM; M-199 containing 0.1% BSA, 25 mM Hepes, and 100 U/ml fungicide), and cut into small (about 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 of tissue; Gibco, Invitrogen Life Technologies, Carlsbad, CA). The details of the tissue dissociation protocol have been previously published [40]. Luteal endothelial cells were separated by a procedure previously described [41, 42]. Briefly, magnetic Tosylactivated beads (Dynal Biotech, Lake Success, NY) were coated with BS-1 lectin (0.15 mg/ml; Vector Laboratories, 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: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). Both BS-1-adhering (endothelial cells) and nonadherent cells (steroidogenic-enriched luteal cells) were collected by this procedure. In this study, the cell population we designated as steroidogenic those cells did not have beads attached, but they represent a heterogeneous population of cells that include fibroblasts, pericytes, lymphoid, and any endothelial cells not removed by our separation procedure. Cell viability and density were determined using Tryptan Blue-exclusion and a hemacytometer; 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 medium 199 (M199) supplemented with 5.0% fetal calf serum (FCS). This initial concentration of FCS in M199 allowed luteal cell attachment to microscope slides. An 80-μl aliquot of the cell suspension was applied to a Cunningham chamber constructed on poly-L-lysine-coated microscope slides [43–45]. 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).

Experiment 1: Potential Role for PKCϵ in $PGF_{2\alpha}$-Initiated Ca^{2+} Signaling

In this experiment, we tested the involvement of PKCϵ in the $PGF_{2\alpha}$-induced calcium signaling in luteal steroidogenic cells isolated from Day 4 and Day 10 corpora lutea. Steroidogenic cells cultured overnight in Cunningham chambers were randomly divided into three groups and treated as follows: 1) M199 containing 50 μg/ml saponin (Sigma, St. Louis MO); 2) M199 containing 50 μg/ml saponin and 1 μM PKCϵ-specific inhibitor, ϵV1–2 (MTA, Stanford, CA); or 3) M199 containing 50 μg/ml saponin and 1 μM PKC conventional-specific inhibitor, betaC2,4 (MTA) for 1 h at 37°C. After saponin permeabilization and inhibitory peptide-delivery, the cells were prepared for single-cell calcium measurements. The tissue culture medium in this portion of the experiment consisted of 127 mM NaCl, 5 mM KCI, 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 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 details for microscope set up for dual wavelength ratio capture and analysis of intracellular calcium concentration have been previously published [40]. For further analysis, the cell responses were represented as changes in the 340:380 nm fluorescence ratios over time. Changes in fluorescence ratio at these two wavelengths have been demonstrated to be due to changes in $[Ca^{2+}]_i$. Microscopic fields were selected using a brightfield image with a 20× objective lens with which cell size and morphology could be determined. Both steroidogenic cells were round and contained lipid droplets; steroidogenic cells identified as large (LLCs) had a diameter of 20 μm or more, while the small steroidogenic cells (SLCs) measured 15 μm or less (Fig. 1). This field selection procedure allowed recording two to three cells per slide. The identified cells were then challenged with experimental medium alone (control) or with experimental medium containing 1000 ng/ml $PGF_{2\alpha}$. This concentration of $PGF_{2\alpha}$was selected because a previous experiment had demonstrated that in the early CL, lower concentrations of $PGF_{2\alpha}$ were not effective in eliciting maximal responses in these cells [40].

Experiment 2: Potential Role for PKCϵ in $PGF_{2\alpha}$-Induced Inhibition of Basal and LH-Stimulated Progesterone Accumulation

This experiment was designed to test the involvement of PKCϵ in $PGF_{2\alpha}$-induced inhibition of basal and LH-stimulated progesterone (P_4) secretion by Day 4 and Day 10 steroidogenic cells. After luteal cell dispersion and purification, steroidogenic cells (1×10^5 cells) were added in small aliquots (100 μl) to wells (Corning 35-mm cell culture clusters; Fisher Scientific Company, Blawnox, PA), containing the following: 1) 1

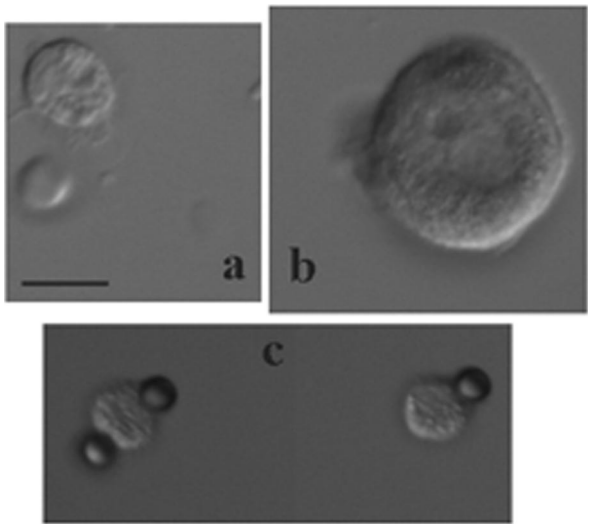

FIG. 1. Representative morphological characteristics of the three cell populations obtained from the dissociated bovine CL. Luteal steroidogenic (**a** and **b**) and endothelial cells were separated using magnetic Tosylactivated beads coated with BS-1 lectin as described in *Materials and Methods.* **a**) A small luteal steroidogenic cell (SLC), which typically had a diameter <20 μm. **b**) A large steroidogenic cell (LLC); these cells typically had a diameter >20 μm. **c**) Two luteal endothelial cells; these cells had one or two magnetic beads attached to their surface. All images were obtained using a ×20 objective lens of an Olympus microscope equipped for Nomarsky microscopy. Bar in (**a**) = 20 μm.

ml M199; 2) 1 ml M199 and saponin (50 μg/ml); 3) 1 ml M199, saponin, and PKCϵ inhibitor (1 μM); 4) 1 ml M199, saponin, and conventional PKC inhibitor (1 μM); 5) 1 ml M199, saponin, and $PGF_{2\alpha}$ (1000 ng/ml); 6) 1 ml M199, saponin, $PGF_{2\alpha}$, and PKCϵ inhibitor; 7) 1 ml M199, saponin, $PGF_{2\alpha}$, and conventional PKC inhibitor; 8) 1 ml M199, saponin, and LH (100 ng/ml); 9) 1 ml M199, saponin, LH, and PKCϵ inhibitor; 10) 1 ml M199, saponin, LH, and conventional PKC inhibitor; 11) saponin, LH, and $PGF_{2\alpha}$; 12) 1 ml M199, saponin, LH, $PGF_{2\alpha}$, and PKCϵ inhibitor; and 13) 1 ml M199, saponin, LH, $PGF_{2\alpha}$, and conventional PKC inhibitor. Each treatment was applied in duplicate to cells from each CL. The cells were incubated for 4 h at 37° C (95% air, 5% CO_2). After incubation, medium free of cells was removed from each well and stored frozen until assayed for measurement of P_4. Measurements of P_4 in the culture media were performed using a radioimmunoassay (RIA) as previously described [46]. The standard curve for this RIA ranged from 10 pg/ml to 800 pg/ml, and the intraassay and interassay coefficients of variation were 9.2% and 12.8%, respectively.

Statistical Analysis

Statistical analyses were performed using the JMP 3.0, a statistical software program from Statistical Analysis Systems [47]. Data are presented as means ± SEM for all experiments. The data for fold increase (340:380 nm ratio) were arcsine transformed to meet the assumptions of normality, and for presentation, all the means were back-transformed accordingly. Three-way analysis of variance (ANOVA) followed by a Tukey-Kramer honestly significant difference test was used to determine statistical significance of fold increase in $[Ca^{2+}]_i$ between PKC inhibitor-treated cells and untreated cells (control). The P_4 data were log-transformed to meet the assumptions of normality, and for presentation, all the means were back-transformed accordingly. One-way ANOVA followed by a Tukey-Kramer honestly significant difference test was used to determine statistical significance differences in P_4 accumulation. A value of $P < 0.05$ was considered significant.

RESULTS

Experiment 1

Morphological characteristics of the LLCs and SLCs identified from steroidogenic-enriched cell populations are shown in Figure 1; SLCs had a diameter <20 μm, LLCs typically had a diameter >20 μm. Using the 20× objective lens typically allowed recording two or three cells per slide. For each cell, any increase in fluorescence ratio that exceeded basal values before stimulation was considered a response. To demonstrate agonist-specificity of the responses, both LLCs and SLCs were stimulated with vehicle media alone. Representative traces of Day 10 LLCs stimulated with vehicle media, $PGF_{2\alpha}$ in the absence or presence of a PKCϵ inhibitor are shown in Figure 2. In Day 10 cells, only 5 of 70 LLCs (7%) and 10 of 80 SLCs (12%) responded to vehicle, and the amplitude of this response was only 1.2-fold ± 0.2-fold in LLCs and 0.8-fold ± 0.5-fold in SLCs over basal values before stimulation. Similarly, in cells from Day 4 corpora lutea, no responders were observed of

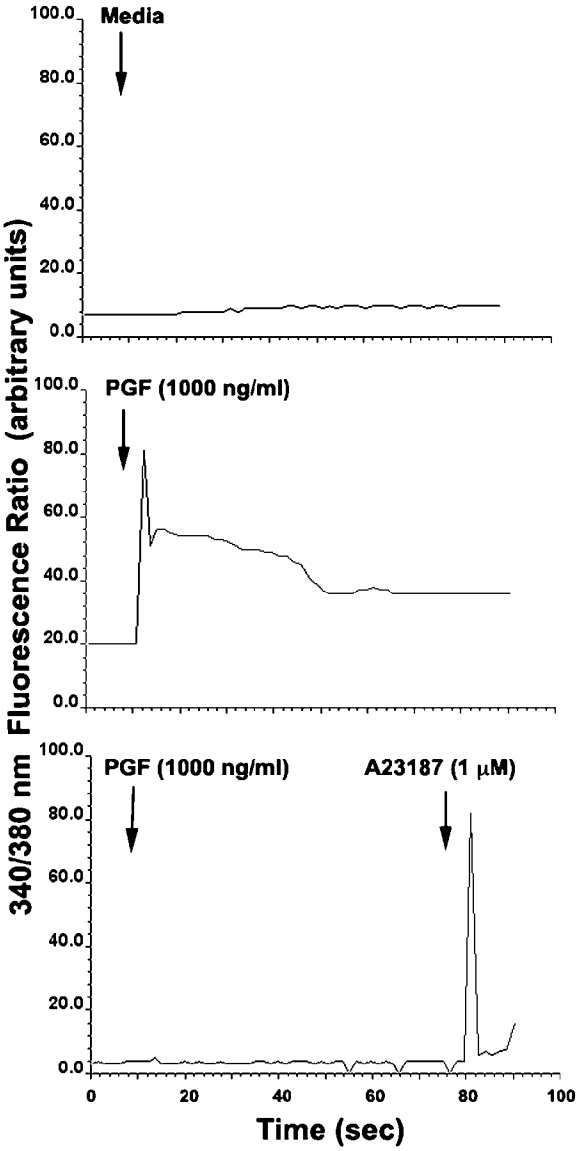

FIG. 2. Specificity of the $PGF_{2\alpha}$-induced Ca^{2+} response and the PKCϵ inhibitor. The cells were isolated from Day 10 bovine corpora lutea and prepared for fura-2 AM imaging of $[Ca^{2+}]_i$ as described in *Materials and Methods.* Data are the relative fluorescence ratio (340:380 nm) over time (in seconds). LLCs were exposed at the indicated time (arrows) to vehicle media (top), to $PGF_{2\alpha}$ alone (1000 ng/ml; middle), and to $PGF_{2\alpha}$ (1000 ng/ml) in the continuous presence of a PKCϵ-specific inhibitor (bottom). At the end of this trace, the cell was exposed to the calcium ionophore, A23187 (1 μM) to demonstrate that even though the PKCϵ inhibitor prevented $PGF_{2\alpha}$from eliciting its typical calcium signal, the ionophore A23178 was able to elicit a calcium response in the same cell.

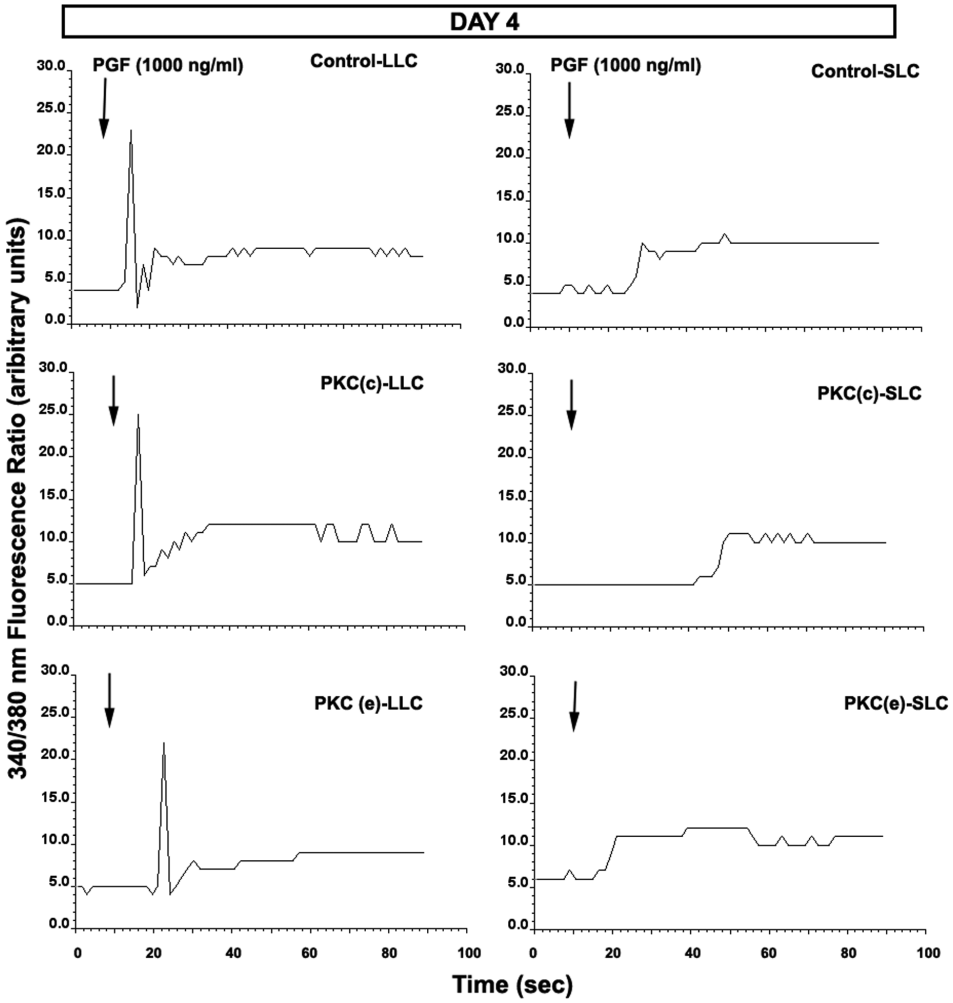

FIG. 3. Representative profiles of the Ca^{2+} responses induced by 1000 ng/ml $PGF_{2\alpha}$ in single, large luteal cells (LLC; left) and small luteal cells (SLC) isolated from Day 4 bovine CL and the effects of PKC isozyme-specific inhibitors on this $PGF_{2\alpha}$-stimulated Ca^{2+} response. The 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 (in seconds). LLCs (left) and SLCs (right) were exposed at the indicated time (arrows) to $PGF_{2\alpha}$ alone (top; Control-LLC and Control SLC), to $PGF_{2\alpha}$ in the continuous presence of a conventional PKC inhibitor (middle; PKC (c)-LLC and PKC (c)-SLC) and $PGF_{2\alpha}$ in the continuous presence of a PKCϵ-specific inhibitor (bottom; PKC (e)-LLC and PKC (e)-SLC). In each panel, a line in the graph represents the trace of a single cell. The units used for the y-axis in the top left panel are different from all other panels in the figure.

80 LLCs tested, while 12 of 90 SLCs (13%) responded with an amplitude of 0.8-fold ± 0.3-fold above the basal level when treated with vehicle media alone.

The concentration of $PGF_{2\alpha}$ used (1000 ng/ml) was effective ($P < 0.05$) in eliciting agonist-specific increases in $[Ca^{2+}]_i$ in LLCs and SLCs (Fig. 3). Representative traces of these $PGF_{2\alpha}$-induced Ca^{2+} responses in LLCs and SLCs collected from Day 4 and Day 10 corpora lutea are shown in Figures 3 and 4. Most of the elicited responses were observed within 45 sec, but there was variability in this aspect of the response. In general, CL development (Day 10 vs. Day 4, compare control LLCs in Figures 3 and 4) and cell type (LLCs vs. SLCs, compare control LLCs with control SLCs in Figures 3 and 4) had significant ($P < 0.001$) effects on the fold increase in the $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$. This is shown clearly in Figure 5, in which the total analysis of the elicited responses is presented.

The fold increase in the $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ in Day 4 LLCs was significantly lower than in Day 10 LLCs (Fig. 5; 4.0 ± 0.6, n = 116 vs. 21.3 ± 2.3, n = 110). Similarly, the fold increase in the $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ in Day 4 SLCs was lower than in Day 10 SLCs (Fig. 5; 1.6 ± 0.2, n = 198 vs. 2.7 ± 0.1, n = 95). On both developmental days examined, the fold increase of the $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ was lower in SLCs than in LLCs (Fig. 5). Although the $PGF_{2\alpha}$-induced fold increase in $[Ca^{2+}]_i$ in Day 4 SLCs was small (1.56 ± 0.2), it was specific and greater ($P < 0.05$) than when SLCs were challenged with vehicle alone (0.8 ± 0.3).

The PKCϵ-specific inhibitor had a significant negative effect on the $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ in both Day 10 LLCs and SLCs (see lower panels in Fig. 4). The presence of the PKCϵ inhibitor drastically reduced the $PGF_{2\alpha}$-elicited responses to 3.5 ± 0.3 (n = 217) and 1.3 ± 0.1 (n = 205) in Day 10 LLCs and SLCs, respectively (Fig. 5). In contrast, treatment with the PKCϵ inhibitor had no effect on the $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ in Day 4 LLCs and SLCs (Fig. 5). In Day 10 cells, a response of great amplitude could still be elicited in inhibitor-treated cells by stimulating them with the calcium ionophore, A23187 (1 μM; Fig. 1, lower panel). This result provides a strong argument in favor of the interpretation that the decrease in the $PGF_{2\alpha}$-induced response was indeed due to the specific effect of the inhibitor on blocking PKCϵ activation and function.

To assess the specificity of PKCϵ involvement in affecting the $PGF_{2\alpha}$-induced calcium signaling, we examined the effect of a PKC inhibitor for all conventional PKCs, beta C2,4. Conventional PKC inhibitor-treated Day 10 LLCs and SLCs responded similarly to control cells without inhibitor (Fig. 5).

Experiment 2

To further analyze the involvement of PKCϵ in the luteolytic actions of $PGF_{2\alpha}$, we measured basal and LH-induced P_4 accumulation in the presence and absence of a PKCϵ-specific inhibitor. Progesterone data from treatments 2, 5, 8, and 11–13 *(Materials and Methods)* from cells iso-

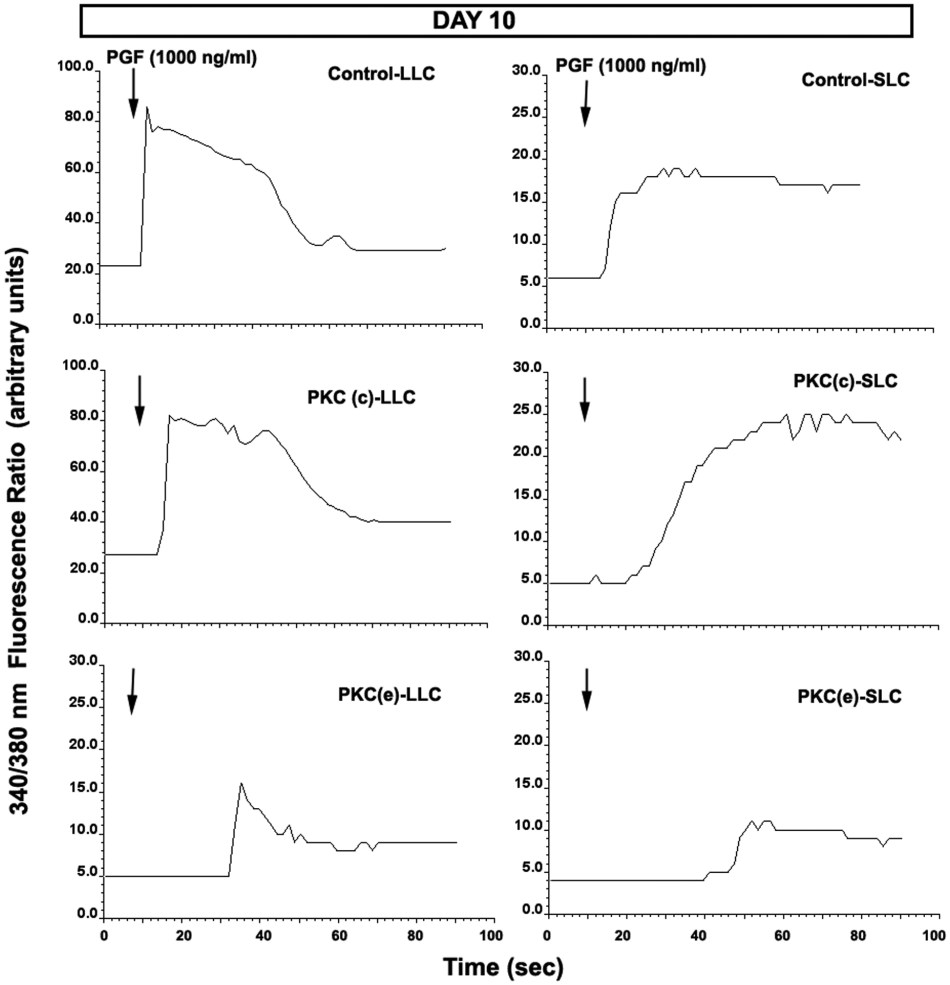

FIG. 4. Representative profiles of the Ca^{2+} response induced by 1000 ng/ml $PGF_{2\alpha}$ in single, large luteal cells (LLC; left) and small luteal cells (SLC; all others panels) cells isolated from Day 10 bovine CL and the effects of PKC isozyme-specific inhibitors on this $PGF_{2\alpha}$-stimulated Ca^{2+} responses. 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 (in seconds). LLCs (left) and SLCs (right) were exposed at the indicated time (arrows) to $PGF_{2\alpha}$ alone (top; Control-LLC and Control SLC), to $PGF_{2\alpha}$ in the continuous presence of a conventional PKC inhibitor (middle; PKC (c)-LLC and PKC (c)-SLC) and $PGF_{2\alpha}$ in the continuous presence of a PKCϵ-specific inhibitor (bottom; PKC (e)-LLC and PKC (e)-SLC. In each panel, a line in the graphs represents the trace of a single cell. The scale units used for the y-axis in the top and middle left panels are different from all other panels in the figure.

lated from Day 10 corpora lutea are shown in Figure 6. No significant differences were observed due to treatment for groups 1, 3, 4, 6, 7, 9, and 10 described in *Materials and Methods;* therefore, the data corresponding to those groups are not shown.

In Day 4 isolated cells, the basal amount of P_4 accumulated in cultures was 14.7 ± 4.3 ng ml 4 h, and none of the treatments had an effect.

Basal P_4 accumulation in cultures of Day 10 steroidogenic cells was three times higher than in Day 4 isolated cells (45.3 ± 10.8 vs. 14.7 ± 4.3 ng ml 4 h, respectively; $P = 0.03$). $PGF_{2\alpha}$ had no effect ($P = 0.27$) on basal P_4 accumulation (Fig. 6). However, LH (100 ng/ml) induced a significant ($P = 0.01$) increase in P_4 accumulation (more than a 3-fold increase) over that observed under basal conditions (Fig. 6). And $PGF_{2\alpha}$ significantly ($P = 0.01$) decreased this effect of LH, reducing it to values below basal conditions (Fig. 6). Progesterone accumulation in luteal cells incubated with LH, $PGF_{2\alpha}$, and the conventional PKC inhibitor was significantly greater than in cells incubated with $PGF_{2\alpha}$ and LH, although not quite as much as incubations with LH, $PGF_{2\alpha}$, and the PKCϵ inhibitor (Fig. 6, $P = 0.02$).

DISCUSSION

This study provides evidence for a differential and specific PKC isozyme function on well-characterized actions of $PGF_{2\alpha}$ in bovine luteal physiology. The results indicate that PKCϵ, an isozyme previously shown to be differentially expressed in Day 10 corpora lutea, has a regulatory role in the $PGF_{2\alpha}$-induced Ca^{2+} signaling, and that this in turn, has consequences on the ability of $PGF_{2\alpha}$ to inhibit LH-stimulated P_4 synthesis at this developmental stage. In other tissues, it is well established that PKC isozymes have very specific functions [48, 49], but in the CL, the biological functions of individual PKC isoforms had not yet been elucidated.

Our single-cell calcium experiments demonstrate that both LLCs and SLCs from Day 10 corpora lutea respond to $PGF_{2\alpha}$with a rise in $[Ca^{2+}]_i$ of greater amplitude than cells from Day 4 corpora lutea. A similar observation was recently reported by Choudhary et al. [40], who studied the full dose-response of LLCs and SLCs to $PGF_{2\alpha}$ as a function of luteal development. Based on this observation, it is strongly suggested that a developmental difference exists in the ability of $PGF_{2\alpha}$ to increase the $[Ca^{2+}]_i$ in both steroidogenic cell types of the bovine CL. That both cell types respond to $PGF_{2\alpha}$ with a rise in $[Ca^{2+}]_i$ is consistent with previous reports that both steroidogenic cell types in bovine CL express functional $PGF_{2\alpha}$ receptors [41, 50, 51]. Although $PGF_{2\alpha}$stimulated a rise in $[Ca^{2+}]_i$ in LLCs and SLCs at both developmental stages, the elicited response in SLCs was of lower amplitude than the one stimulated in LLCs. This latter observation agrees with the responses elicited by $PGF_{2\alpha}$ in LLCs and SLCs in the study by Choudhary et al. [40]. In earlier studies [5, 52–54], $PGF_{2\alpha}$-induced Ca^{2+} responses were observed only in LLCs. These difference could be due to species differences between cows and sheep

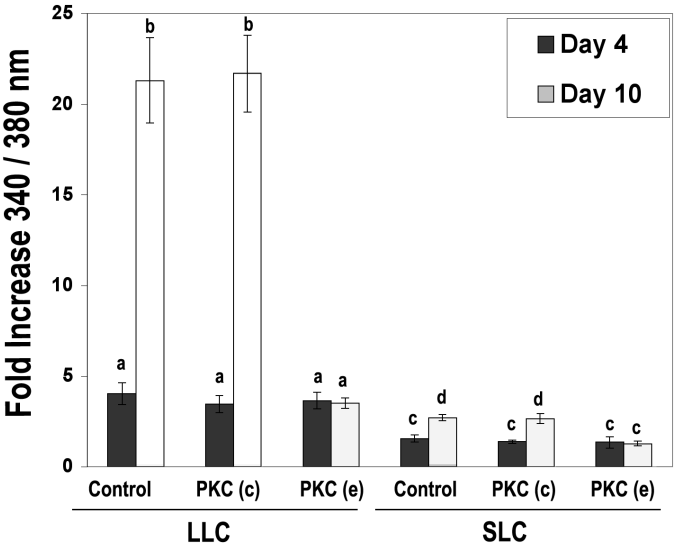

FIG. 5. Summary of the effects of conventional PKC [PKC (c)] and PKCϵ inhibitors [PKC (ϵ)] on the $PGF_{2\alpha}$-stimulated rise in $[Ca^{2+}]_i$ in Day 4 and Day 10 LLCs and SLCs. The cells were isolated and prepared for fura-2 AM imaging of $[Ca^{2+}]_i$ as described in *Materials and Methods.* Cells were pretreated with either no inhibitors (control cells) or with PKC(c)- or PKCϵ-specific inhibitor. Values are presented as the mean ± SEM of the fold increase in $[Ca^{2+}]_i$ induced by $PGF_{2\alpha}$ (1000 ng/ml) from basal values observed before the stimulation with $PGF_{2\alpha}$. Statistical comparisons were made within cell type, developmental stage, and treatment; different letters on top of bars denote significantly different values ($P < 0.05$ across treatments depicted by the bars; for Day 4, n = 116, 224, 225, 198, 189, and 208; and for Day 10, n = 110, 202, 217, 95, 182, and 205).

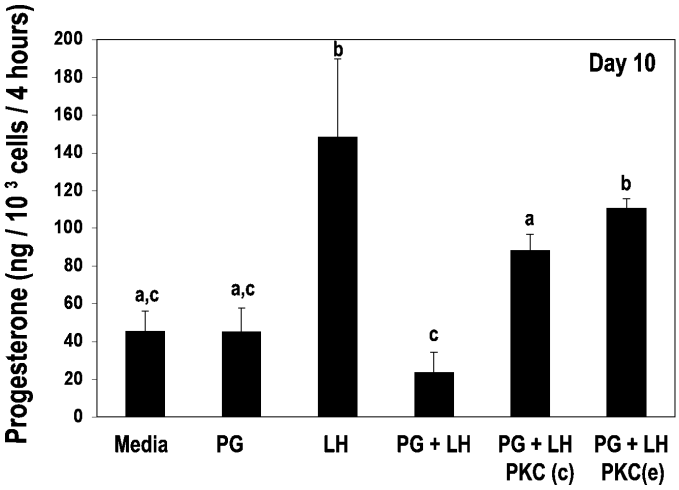

FIG. 6. Effects of conventional PKC [PKC (c)] and PKCϵ inhibitors [PKC (ϵ)] on $PGF_{2\alpha}$-actions on the basal and LH-stimulated progesterone accumulation in cultures of steroidogenic cells collected from Day 10 bovine CL. Progesterone accumulation was determined in culture media after 4 h of incubation in the following treatments: media alone (Media); $PGF_{2\alpha}$ (PG; 1000 ng/ml); $PGF_{2\alpha}$ and LH (PG + LH; 1000 ng/ml and 100 ng/ml, respectively), $PGF_{2\alpha}$, LH, and inhibitor conventional PKC [PKC (c) PG + LH; 1000 ng/ml, 100 ng/ml, and 1 μM, respectively]; and $PGF_{2\alpha}$, LH, and PKCϵ inhibitor [PKC (ϵ) PG + LH; 1000 ng/ml, 100 ng/ml, and 1 μM, respectively]. As explained for experiment 2 in *Materials and Methods,* all these treatments also contained saponin (50 μg/ml). Data are presented as the mean ± SEM of four (Day 10) individual replicates (cows). Statistical comparisons were made across cell type, developmental stage, and treatment; different letters on top of bars denote significantly different values, $P < 0.05$.

[5, 52], or to technical differences; a cell population approach was used in one of those studies [54], whereas a single-cell approach was used in the present study for the measurement of $[Ca^{2+}]_i$. Differences in regulation of $[Ca^{2+}]_i$ homeostasis in LLCs and SLCs have been documented previously [51, 53–56] in ovine and bovine corpora lutea. This difference between LLCs and SLCs might explain the differences observed here with regard to the responses elicited by $PGF_{2\alpha}$ in LLCs and SLCs. Alila et al. [57] reported that LH induced a rapid increase in $[Ca^{2+}]_i$ that differed both in magnitude and profile between LLCs and SLCs [57]. Of interest, in the study by Alila et al., it was the SLCs that responded with greater amplitude when stimulated by LH [57].

The PKCϵ-specific peptide inhibitor (ϵV1–2) used in this study has been demonstrated to block the interaction of PKCϵ with its specific RACK in an effective manner, thereby preventing its translocation and its function [10, 11, 29–31]. The interpretation that this was also true in our study is supported by the observation that this inhibitor greatly decreased the amplitude of the $PGF_{2\alpha}$-induced Ca^{2+} response in cells isolated from Day 10 corpora lutea. As expected, in Day 4 corpora lutea in which PKCϵ is expressed at very low levels, blocking PKCϵ action had no effect on the magnitude of the $PGF_{2\alpha}$-induced Ca^{2+} signal, which was of low amplitude to begin with. Therefore, based on our data it is suggested that PKCϵ might have a regulatory role in the $PGF_{2\alpha}$-induced Ca^{2+} signal in both cell types of the midluteal phase corpora lutea. Furthermore, we propose that the developmental difference in the ability of $PGF_{2\alpha}$ to increase the $[Ca^{2+}]_i$ in both steroidogenic cells types of the Day 4 vs. Day 10 bovine CL is due to the lower expression of PKCϵ at this stage [28].

Of interest, $PGF_{2\alpha}$ inhibited LH-stimulated P_4 accumulation only in Day 10 luteal cells. This developmental association of the inhibitory action of $PGF_{2\alpha}$ agrees with the report by Choudhary et al. [40]; however, in that study, the inhibitory actions of $PGF_{2\alpha}$ were observed on basal and LH-stimulated P_4 accumulation. This discrepancy is most likely due to the permeabilization protocol used in the present study. Although saponin did not have any effect on P_4 accumulation on medium-treated control cells (data not shown), the effects of LH and of $PGF_{2\alpha}$ may have been affected due to increased digitonin-mediated permeability. This interpretation is supported by the observation that in the present study, LH-stimulated P_4 accumulation occurred only in Day 10 luteal cells, whereas Choudhary et al. [40] reported an LH-stimulated P_4 accumulation in Day 4 and Day 10 luteal cells. However, more importantly, the PKCϵ inhibitor greatly reduced the ability of $PGF_{2\alpha}$ to inhibit LH-stimulated P_4 accumulation. Therefore, at the level of P_4 accumulation, PKCϵ might also have some regulatory role in the $PGF_{2\alpha}$-induced inhibition of P_4 accumulation in cultures of cells isolated from bovine corpora lutea that had acquired luteolytic responsiveness to $PGF_{2\alpha}$. Our results do not allow us to discern the precise link between calcium signal and P_4 synthesis, but clearly, Figure 6 illustrates that both conventional as well as ϵ PKC isozymes are involved in mediating the inhibitory actions of $PGF_{2\alpha}$. However, on the basis of calcium and progesterone data presented here, we propose that once the CL has acquired the ability to respond to inhibitory actions of $PGF_{2\alpha}$, PKCϵ it is the isozyme that significantly mediates the $PGF_{2\alpha}$-induced calcium signal, and that this in turn, via conventional PKC isozymes, mediates the inhibition by $PGF_{2\alpha}$of LH-stimulated inhibition of P_4 accumulation. This interpretation is supported by the observation in experiment 2 in which P_4 accumulation in luteal cells incubated with LH, $PGF_{2\alpha}$, and

the conventional PKC inhibitor was significantly higher than in cells incubated with LH and $PGF_{2\alpha}$, although not quite as much as with LH, $PGF_{2\alpha}$, and the inhibitor of PKCϵ.

The role of PKC in luteal physiology is quite controversial; this may be related to differences in the mechanism used to activate PKC (PMA or $PGF_{2\alpha}$); incomplete specificity of PKC inhibitors such as H-7, W-7, GF109203X, and staurosporine used in previous studies; the time and dose of agonist used; the tissue used (luteinized granulosal cells, different developmental stages of CL); species differences; and so on. For example, PMA activates all PKC isozymes, whereas hormones such as $PGF_{2\alpha}$ may activate only a subset of the PKC array expressed in the cells. Consequently, a variety of studies indicate that PKC stimulates, has no effect, or inhibits P_4 synthesis in luteal tissue [58–62]. Nevertheless, it is clear that an involvement of PKC in the negative regulation of P_4 synthesis in vivo has been demonstrated [63]. Furthermore, Wiltbank et al. demonstrated that $PGF_{2\alpha}$ has a direct antisteroidogenic effect on both LLCs and SLCs that is mediated through the PKC second-messenger pathway [64].

As expected, neither $PGF_{2\alpha}$ nor PKCϵ inhibitor had any effect on P_4 accumulation in Day 4 cultured steroidogenic cells. Earlier studies using the PKC antagonist W-7 have shown an inhibition of both basal and hormone-stimulated P_4 synthesis in SLCs and LLCs [57]. However, we did not observe any effect of $PGF_{2\alpha}$, conventional PKC inhibitor, or PKCϵ inhibitor on basal P_4 accumulation in cells from either day, which may be due to differences in experimental procedures as discussed above. Also, our study did not identify whether the effect of $PGF_{2\alpha}$ or PKCϵ inhibitor on the LH-stimulated P_4 accumulation occurs in both LLCs and SLCs or in LLCs only.

LLCs are suggested to be the potential target of the inhibitory (luteolytic) effect of $PGF_{2\alpha}$, whereas SLCs are said to be responsible for the (stimulatory) luteotropic effect of $PGF_{2\alpha}$ in the bovine CL [64]. However, we observed that $PGF_{2\alpha}$-induced Ca^{2+} responses were significantly decreased in both LLCs and SLCs when PKCϵ was blocked. The regulatory role of PKCϵ in the $PGF_{2\alpha}$-induced Ca^{2+} signal might have different cellular consequences in LLCs and SLCs. We do not know whether both steroidogenic cell types express the same PKC isozymes. Potentially, both PKC activity and substrate availability could bring about differences in regulation of steroidogenesis by PKC. In ovine steroidogenic cells it has been reported that PKC activity and available protein substrates displayed quantitative and qualitative differences between SLCs and LLCs, and that differences in the regulation of steroidogenesis between these cells might be due to these differences [65]. It has been proposed by Braden et al. that the cytotoxic effects of $PGF_{2\alpha}$ may be due to sustained elevation of $[Ca^{2+}]_i$ [66]. In this regard, there is evidence for both extracellular and intracellular calcium contributions to the $PGF_{2\alpha}$-induced Ca^{2+} response [54]. Our studies do not allow an assessment of whether PKCϵ is modulating the effect of $PGF_{2\alpha}$ on intracellular Ca^{2+} mobilization, Ca^{2+} influx, or both.

An interesting suggestion in the literature is that there may be an appropriate threshold of $[Ca^{2+}]_i$ that is required to support P_4 synthesis [54]. An alteration in the free calcium concentration could be the intracellular second-message that mediates the luteolytic actions of $PGF_{2\alpha}$ [53]. In this context, the differential expression of PKCϵ as a function of development [28] and the possibility that PKCϵ has an important regulatory role in the $PGF_{2\alpha}$-induced Ca^{2+} signal can be interpreted as being of great physiological significance. The expression of PKCϵ and its activation by $PGF_{2\alpha}$ may shift the $[Ca^{2+}]_i$ signal from a luteotropic threshold to a luteolytic one in Day 10, thereby playing a role in the differential sensitivity of the CL to $PGF_{2\alpha}$.

Blocking the action of all conventional PKC isozymes (α, βI, and βII) expressed in the bovine CL [28] at both development stages had no effect on the $PGF_{2\alpha}$-induced Ca^{2+} signal. This supports the interpretation that these actions were specific for PKCϵ. As mentioned earlier, conventional PKC isozymes also appear to be involved in mediating the inhibitory actions of $PGF_{2\alpha}$ on LH-stimulated P_4 accumulation. However, the exact roles of these PKC isozymes are still unknown.

In summary, PKCϵ appears to have a key regulatory role in the calcium signaling initiated by $PGF_{2\alpha}$, and this, at least in part, appeared to antagonized the inhibitory effect of $PGF_{2\alpha}$ on LH-stimulated P_4 accumulation in cultures of Day 10 luteal steroidogenic cells. Therefore, we propose that the differential ability of both LLCs and SLCs to exhibit a $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ as a function of development is due to the differential expression and activation of this isozyme in Day 10 corpora lutea. The inability of $PGF_{2\alpha}$ to decrease P_4 secretion in Day 4 corpora lutea may be related to the absence of this PKCϵ at this developmental stage of the CL. Thus, based on the above observations, we propose that expression and activation of PKCϵ in the midphase bovine CL, shifts the $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ response to a threshold that allows activation of conventional PKC isozymes, and this in turn, decreases P_4 accumulation characteristic of luteal regression. However, other mechanisms such as the tonic inhibition of P_4 accumulation by ET-1 [40], and nitric oxide [67, 68] may act in an additive fashion with $PGF_{2\alpha}$ to cause luteal regression during the mid- to late-luteal phase of the bovine CL.

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