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Participation of specific PKC isozymes in the inhibitory effect of ET-1 on progesterone accumulation in cells isolated from early- and mid-phase corpora lutea

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Abstract

Expression of PKC α, β I, β II, ε and μ has been demonstrated in the whole bovine CL with PKC ε being differentially expressed as a function of development. In experiment 1 we have investigated the amount of mRNA encoding PKC ε at different stages of luteal development (days 1, 4, 10 and 17). In experiment 2, the cellular source of luteal PKC isozymes was determined. Enriched steroidogenic (SC) and endothelial (EC) cells from day-10 CL were used to examine this question by Western blot analysis and immuno-histochemistry. In experiment 3, Western blot analysis was used to examine the ability of ET-1 to activate luteal PKC isozymes in day-10 CL. In experiment 4, the role of luteal PKC isozymes in the ET-1 mediated inhibition of P_4 accumulation in steroidogenic cell cultures from day-4 and day-10 CL was examined. Abundance of PKC ε mRNA gradually increased from day-1 to -10 with no further increase on day-17. In experiment 2, PKC ε was exclusively detected in SC (LLC and SLC). In contrast, PKC α, β I and β II were detected in both SC and EC, with EC expressing higher amounts of PKC isozymes. In day-10 CL, ET-1 induced cellular redistribution of PKC α, β I, ε but not β II. Inhibitors specific for conventional PKC isozymes as well as PKC ε were able to negate the inhibitory

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effects of ET-1 on P4 accumulation in the day10 CL. In the day-4 CL, the inhibitory effect of ET-1 might be mediated via conventional PKC. Thus, an exclusive presence of PKC ε in luteal steroidogenic cells, its higher expression along with the ability of ET-1 to stimulate its activation in day-10 CL strongly suggests that this PKC isoform may play an important regulatory role in decreasing P_4 during luteal regression. Inhibition of P_4 by ET-1 in the early CL may be mediated via conventional PKC isozymes.
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1. Introduction

Progesterone (P_4) produced by the corpus luteum (CL) is necessary for establishing and maintaining pregnancy [1]. If pregnancy does not ensue, the CL enters a regression or luteolytic process during which it loses the capacity to produce P_4 and undergoes structural involution [2]. Regulation of P_4 production as well as luteal regression involves interactions between luteal endothelial and steroidogenic cells [3,4].

In mammals, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the most important hormone associated with luteal regression [1,2] and based on this knowledge, it has been widely used for the purpose of estrous synchronization in farm animals. However, despite its widespread application, the mechanisms by which $PGF_{2\alpha}$ induces luteal regression are not completely understood. For instance, the CL is resistant to the luteolytic actions of $PGF_{2\alpha}$ prior to day 6 of the estrous cycle, rendering prostaglandin treatment drastically less effective before that time [5].

Two important intracellular mediators of the luteolytic actions of $PGF_{2\alpha}$ in luteal steroidogenic cells [2] are protein kinase C (PKC) and the cytoplasmic concentration of calcium ions ($[Ca^{2+}]_i$). PKC is a family of serine-threonine kinases that exist in at least 11 closely related isozymes [6]. The array of PKC isozymes expressed in whole bovine CL includes α, βI, βII, ε and μ [7–9]; and it has been demonstrated that the amount of PKC ε expressed in the day-10 CL is greater than in the day-4 CL [9]. The latter observation led us to propose that differential expression of PKC ε as a function of development could play a role in the $PGF_{2\alpha}$-induced luteal regression [9,10].

Studies in different species and cell types indicate that differences in co-activator requirements for each PKC isozyme as well as distinct cellular localization contribute to isozyme functional specificity [11–13]. The cellular source(s) for each PKC isozyme expressed in the CL has not been examined and consequently, our ability to approach several strategies to determine a specific role for each PKC isozyme in luteal physiology is limited. For instance, available strategies to down- or up-regulate expression of a given PKC isozyme for assessing its function require knowledge of the normal temporal and spatial (cellular source) expression of that isozyme.

Several authors collectively have indicated that endothelin-1 (ET-1), secreted by luteal endothelial cells, plays a role in luteal regression [3,4,14–21]. While some investigators have suggested that ET-1 is a mediator of the luteolytic actions of $PGF_{2\alpha}$ [3,14,16,18], our own data have indicated that ET-1 although a tonic inhibitor of P_4 synthesis, is not necessarily a mediator of $PGF_{2\alpha}$ actions [22]. The intracellular mediator(s) of ET-1 actions in luteal regression is not yet known, however, actions of ET-1 in luteal cells [20] as well as in

granulosal cells [23] are known to involve, at least in part, the participation of phospholipase C (PLC), inositol phosphates and intracellular calcium. Moreover, little is known about the intracellular mediators or mechanism(s) involved in the inhibition of P_4 synthesis by ET-1 in the CL.

Thus, the aims of these studies were: (1) to determine the temporal expression of mRNA encoding PKC ε gene as a function of luteal development; (2) to identify the cellular source for each luteal PKC isozyme; (3) to investigate the ability of ET-1 to activate in vitro, the different luteal PKC isozymes in the day-10 CL; and (4) to determine the roles of luteal PKC isozymes in the ET-1 mediated inhibition of P_4 accumulation in steroidogenic cell cultures from day-4 and day-10 CL.

2. Materials and methods

2.1. Luteal tissue collection

Bovine CL were collected as previously described [9,10,22]. Briefly, behavioral estrus was determined in non-lactating beef cows. The day of standing estrus was designated as Day-0 [24] and after two cycles, day-1, day-4, day-10 and day-17 CL were collected by ovariectomy (day-1 and -4) or blunt dissection (day-10 and -17) via supravaginal incision under epidural anesthesia [9,10,22]. For the epidural anesthesia, 6–9 ml 2% lidocaine were administered for cows weighing 450–700 kg (Butler Company, Columbus, OH). The CL or ovary was collected into ice-cold phosphate-buffered saline (PBS) pH 7.4 and transported to the laboratory within 15 to 30 min after collection. The West Virginia University Animal Care and Use Committee reviewed and approved the protocol for the tissue collection (ACUC #01-0809).

2.2. Luteal cell dispersion and purification

Luteal cell dispersion was performed as previously described [10,22]. Briefly, the CL tissue was dissociated in cell dispersion medium (CDM, M-199 containing 0.1% BSA, 25 mM Hepes, 100 U/ml fungicide) containing collagenase type IV (Gibco, Invitrogen Life Technologies, Carlsbad, CA, 420 U/ml/g of tissue). The dispersed luteal cells were then suspended in 1% PBS, mixed with magnetic tosylactivated beads (Dynal Biotech, Lake Success, NY) coated with BS-lectin 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 non-adherent cells (steroidogenic luteal cells) were then collected by this procedure. Cell viability and density were determined using Trypan Blue exclusion and a hemacytometer; cell viability was greater than 96%.

2.3. Semi-quantitative reverse transcriptase-PCR

Total RNA was isolated with Trizol reagent according to the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD). The isolated RNA was quantified spectroscopically at

260 nm and used in a one-step semi-quantitative reverse transcriptase-PCR (RT-PCR, Qiagen, Valencia, CA) for PKC ε and GAPDH (reference gene). The identity of the primers for PKC ε were those published elsewhere [25]; sense 5′-AGCTTGAAGCCCACAGCCTG-3′; antisense 5′-CTTGTGGCCGTTGACCTGATG-3′. Primers for GAPDH amplification have been published [19,26], sense 5′-TGTTCCAGTATGATTCCACCC-3′; antisense 5′-TGTTCCAGTATGATTCCACCC-3′. The specificity for these primer sets have been documented [19,25,26], the primer specificity for amplification of PKC ε mRNA was confirmed here by using the nucleotide database of National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed) with the BLAST software. The RT-PCR assay conditions were as follows: 50 °C for 30 min for reverse transcription reaction, 95 °C for 15 min for inactivation of RT enzyme, and then for PCR cycles consisted of 95 °C for 50 s for denaturing, 58 °C for 30 s for annealing, 72 °C for 1 min for extension and a final extension of 5 min at 72 °C. The RT-PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and viewed using the Fluro-S MultiImager (Bio-Rad Laboratories, Hercules, CA). Data were collected using densitometric analysis of Quantity One quantification software package (Version 4, Bio-Rad Laboratories, Hercules, CA). The intensity of the signal corresponding to PKC ε was standardized by the corresponding intensity of GAPDH control in that sample.

2.4. Semi-quantitative Western blotting

Proteins from separated luteal cell populations were isolated by placing the cells in homogenization buffer (containing 20 nM Tris–HCL, 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (Eastman Kodak Company, Rochester, NY), 20 μg/ml leupeptin, and 20 mM 2-mercaptoethanol (Gibco) and homogenizing them using an ultrasonic homogenizer (Cole Palmer Instrument Company, Chicago, Ill). The cell-homogenate was centrifuged at $100 \times g$ for 10 min to separate coarse cellular particles. Protein concentration in the samples was determined by Bio-Rad assay (Hercules, CA) with BSA (Gibco) as standard. Ten μg/lane of sample protein was used for semi-quantitative western blot analysis as previously described [9]. The following primary antibodies were used in this experiment: mouse anti-actin monoclonal antibody (used at a dilution of 1:3000 (v/v); Chemicon International Inc., Temecula, CA); four PKC isozyme specific (α, βI, βII and ε) affinity purified polyclonal antibodies (used at a dilution 1:1000 (v/v); Gibco). The following secondary antibodies were used in this experiment: anti-rabbit (1:5000 (v/v); Amersham Pharmacia Biotech, Piscataway, NJ) and anti-mouse (1:30,000 (v/v); Gibco) horseradish peroxide-conjugated antibodies. Validation of the semi-quantitative western blot analysis and stripping conditions have been determined previously [9]. The intensity of the signal corresponding to the protein of interest was standardized by the corresponding intensity of the actin control in that sample. Normalization of data allowed us to estimate, in a semi-quantitative manner, the amounts of protein in the samples of interest.

2.5. Immunohistological assay

Frozen CL ($n = 4$) were sectioned at 10 μM thickness using a Richard-Allan Scientific cryostat (Series HM505 E, Fischer Scientific, Pittsburgh PA) and thawed-mounted on

microscope slides. These sections were used for immuno-histochemistry. The tissue sections were fixed with ice-cold acetone for 15 min, followed by 3× wash in Tris–HCl –buffered saline (TBS) for 10 min each. Blocking of non-specific binding sites was accomplished with 5% normal goat serum (NGS, Sternberger Monoclonals Inc., Baltimore, MD) in TBS for 30 min in a moist chamber at room temperature. Sections were then incubated with PKC ε specific primary (Gibco) antibody at a dilution of 1:500 (v/v) in 1% NGS-TBS at 4 °C overnight in a moist chamber. The next day the slides were washed 3× in TBS for 10 min each followed by incubation with secondary antibody, anti rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) at a dilution of 1:200 (v/v) in 1% NGS-TBS at room temperature for 30 min in a moist chamber. Endogenous peroxidase activity was reduced by incubating the sections in a solution of 3% hydrogen peroxide in methanol at room temperature for 30–45 min. Tissue sections were then washed 2× in TBS for 10 min each and incubated with rabbit PAP complex (Sternberger Monoclonals Incorporated, Baltimore, MD) at a dilution of 1:200 (v/v) in 1% NGS-TBS at room temperature for 30 min. The PAP solution was removed and slides were washed 3× in TBS for 10 min each. The CL sections were incubated with the substrate, 3,3′-diaminobenzidine chromogen solution (DAB, prepared according to the manufacturers instructions; Biogenex, San Ramon, CA) at room temperature for 3, 5 and 10 min. Slides incubated for 5 min showed the best signal to noise ratio and 5 min was selected as the incubation time to be used in all sections of the four CL used for analysis. Slides were allowed to dry at room temperature and cover slips were placed using an aqueous mounting medium (Gel/Mount Biomedia Corp. Foster City, CA). These slides were later observed under an Olympus PROVIS AX70 microscope (Olympus America Inc., Melville, NY). Each treatment was performed in three consecutive sections on different slides. Pre-incubation of the primary antibody with excess antigenic peptide has previously [7,32] validated the specificity of the primary antibody. The specificity of the immunohistological detection was further determined by: (1) omission of primary antibody; (2) omission of secondary antibody; and (3) omission of PAP antibody. The slides were examined in the microscope for the presence/absence of specific brown color accumulation indicating immunoreactivity.

2.5.1. Experiment 1: Temporal expression of mRNA encoding PKC ε during luteal development

The temporal expression of mRNA encoding PKC ε was examined using a one-step semi-quantitative reverse transcriptase-PCR assay with RNA samples isolated from day-1 ($n = 3$), -4 ($n = 3$), -10 ($n = 4$) and -17 ($n = 3$) CL samples. The amount of total RNA in the assay for each developmental day was adjusted to 200 ng per reaction and the number of cycles was optimized to 26 and 40 for amplification of GAPDH and PKC ε, respectively. Subsequently, as under these conditions, the amount of mRNA encoding PKC ε was lowest in the day-1 samples, the RT-PCR assay also was performed with different amounts of total RNA (100, 300 and 500 ng) for samples with lowest (day-1) and highest (day-10) amounts of PKC ε mRNA. The validity of the conditions used here for the semi-quantitative RT-PCR has been previously demonstrated (19, 26) and was also confirmed in this study.

2.5.2. Experiment 2: Cellular source of luteal PKC isozymes

The cellular source for each luteal PKC isozyme was examined using a semi-quantitative Western blot analysis of proteins isolated from enriched steroidogenic and endothelial cell

populations collected from day-10 CL ($n = 3$) and by an immunohistological detection of PKC ε on luteal sections prepared from frozen tissue collected on day-10 of the ovarian cycle ($n = 4$).

2.5.3. Experiment 3: Ability of ET-1 to activate luteal PKC isozymes

Day-10 CL were dissected free of any connective tissue and then cut into small 1 mm^3 fragments just before the experiment was initiated. The CL fragments were added to disposable culture tubes (Fisher Scientific, Pittsburgh, PA) containing MEM-HEPES (Gibco Brl, Life Technologies) alone or MEM-HEPES containing 100 nmol ET-1. The tissue and media were separated by centrifugation after 10 min, snap frozen in liquid nitrogen and stored at −80 °C. The tissue was later pulverized and homogenized in homogenization buffer. The homogenized tissue was centrifuged at $1000 \times g$ for 2 min at 4 °C to remove coarse tissue particles including nuclei. This supernatant was used for subcellular fractionation by differential and discontinuous sucrose gradient centrifugation as previously described (9). Protein concentrations in the cytosolic and membrane fractions were determined using a Bio-Rad assay with BSA as standards. 10 μg/lane of sample proteins were analyzed by a semi-quantitative Western blotting as previously described [4,9]. Data are presented as the ratios of actin-corrected optical density (O.D.) detected for the PKC isozyme in the membrane fraction to the corrected O.D. for the same isozyme in the cytosolic fraction (M/C).

2.5.4. Experiment 4: Role of luteal conventional PKC and PKC ε in the ET-1 mediated inhibition of P_4 accumulation in day-4 and day-10 luteal steroidogenic cells

The aim of this experiment was to test the involvement of conventional PKC (α, βI, βII) and novel PKC (ε) isozymes in ET-1 induced inhibition of P_4 accumulation [22] in day-4 ($n = 4$) and day-10 ($n = 3$) luteal steroidogenic cells. This experiment was performed as previously described [10]. Briefly, enriched populations of steroidogenic cells (1×10^3 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 ml M199 and the permeabilizing agent saponin; (2) 1 ml M199, saponin and LH (100 ng/ml); (3) 1 ml M199, saponin and ET-1 (100 nM); (4) 1 ml M199, saponin, LH and ET-1; (5) 1 ml M199, saponin, LH, ET-1 and conventional PKC inhibitor (1 μM) and (6) 1 ml M199, saponin, LH, ET-1 and PKC ε inhibitor (1 μM). 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 stored frozen until assayed for P_4 by radioimmunoassay (RIA) as previously described [10,22]. The standard curve for this RIA ranged from 10 to 800 pg/ml, and the intra-assay and inter-assay coefficients of variation were 9.2 and 12.8%, respectively.

2.6. Statistical analysis

Statistical analyses were performed using JMP, a statistical software program from Statistical Analysis Systems (SAS, Cary, NC [27]). The results were expressed as the mean ± S.E.M. Two-way ANOVA followed by Tukey–Kramer honestly significant difference test was used to determine statistically significant differences between amounts of PKC ε mRNA among different luteal developmental stages or PKC isozymes and cell types

or PKC isozymes and ET-1 treatment (experiments 1 and 2). The data from experiment 4 was analyzed by one-way ANOVA and a comparison of all means was made; Tukey–Kramer honestly significant difference was used to determine statistically significance differences in P_4 accumulation. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Experiment 1

Fig. 1A shows the profiles for the amount of luteal mRNA encoding PKC ε at the four luteal developmental stages examined. Abundance of PKC ε mRNA gradually increased from day-1 to day-10 CL. There were increases from day 1 to day 4 and from day 4 to day 10 ($P < 0.05$). No further increase in the amount of mRNA encoding PKC ε was observed in day-17 CL; the amount of PKC ε mRNA in day 17 was similar to that of day 10.

A representative picture of the RT-PCR products obtained using primers for PKC ε and GAPDH from day-1 and day-10 samples is shown in Fig. 1B. The approximate size of the amplified PKC ε and GAPDH fragments were 480 and 900 bp, respectively. These results agree with the size of the amplified fragments previously reported in studies using these primers [19,25,26].

When the RT-PCR assay was performed using increasing amounts of RNA/reaction (100, 300 and 500 ng/reaction), in the day-1 samples, an amplified fragment corresponding to the PKC ε mRNA was obtained only when 300 and 500 ng/reaction were used. In contrast, when the RNA from day-10 samples was used, all three amounts of RNA were effective for amplifying the cDNA fragment corresponding to PKC ε (Fig. 1B). This quantitative relationship is shown in Fig. 1C. Furthermore, the abundance of the amplified fragment in the day-1 samples was always lower than that amplified in the day-10 samples (Fig. 1C). This clearly indicates that the amplified product was a function of the amount of mRNA template and that this in turn, was expressed at lower concentrations as a function of luteal development as depicted in the summarized data in Fig. 1A. Furthermore, the experiment using real-time PCR assay corroborated the observation that the abundance of the RNA encoding PKC ε in day-1 samples was lower than in day-10 samples (data not shown).

3.2. Experiment 2

Fig. 2A shows a representative blot for PKC α, ε and actin obtained from the enriched luteal cell populations tested. PKC ε was detected exclusively in the steroidogenic cells (Fig. 2A) in contrast to PKC α that was detected in both cell populations. Fig. 2B shows the summary of the amount of protein corresponding to PKC α, βI, βII and ε in separated steroidogenic (LLC and SLC) and endothelial cells from day-10 CL. Although, both steroidogenic and endothelial cells expressed PKC α, βI and βII, their amounts were significantly lower in endothelial cells than in steroidogenic cells ($P < 0.05$). Furthermore, in steroidogenic cells, all PKC isozymes were expressed in equal amounts; whereas in endothelial cells, α and βII isozymes were expressed in higher amounts than βI ($P < 0.05$).

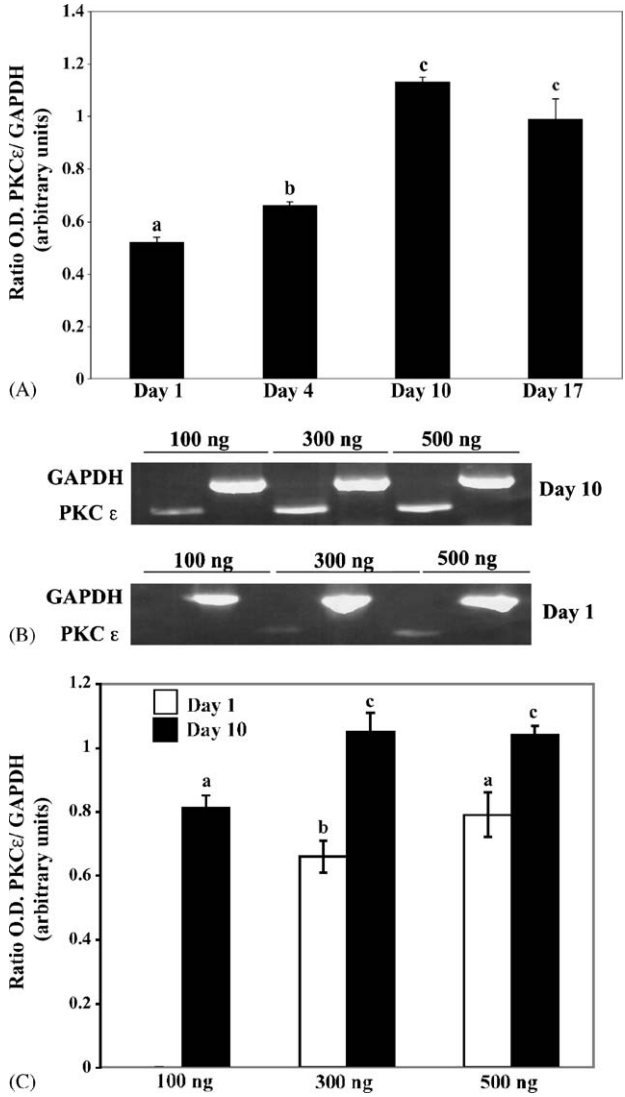

Fig. 1. A Semi-quantitative analysis of the amounts of mRNA encoding PKC ε as a function of luteal development. Total RNA (200 ng/reaction) isolated from day 1 ($n = 3$), day 4 ($n = 3$), day 10 ($n = 4$) and day 17 ($n = 3$) CL were used for the RT-CR assay. Data are presented as the mean ± S.E.M. of densitometric analysis of PKC ε relative to GAPDH mRNA; values with different letters denote statistically significant differences ($P < 0.05$). Panel B shows the representative RT-PCR products obtained by using different amounts of RNA/reaction (100, 300 and 500 ng) isolated from day 1 (lower panel) and day 10 (upper panel) CL using GAPDH and PKC ε specific primers. The sizes of the amplified products for GAPDH and PKC ε were 900 and 480 bp, respectively. Panel C shows the semi-quantitative analysis of amplified PKC ε mRNA as a function of different amounts (100, 300 and 500 ng) of RNA/reaction from day 1 ($n = 3$) and day 10 ($n = 3$) CL. Data are presented as the ±S.E.M. of densitometric analysis of PKC ε relative to GAPDH mRNA; values with different letters denote statistically significant differences ($P < 0.05$).

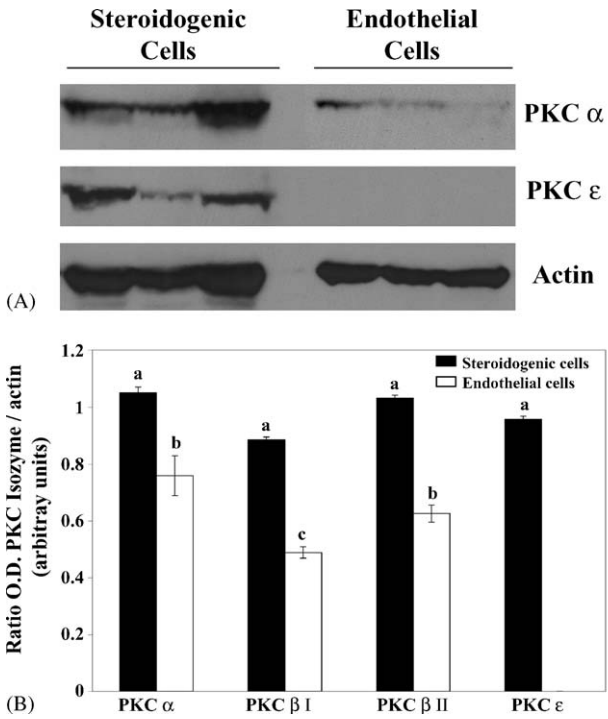

Fig. 2. Panel A shows a representative Western blot of PKC α, PKC ε and actin from enriched steroidogenic and endothelial cells collected from day-10 CL ($n = 3$). The middle panel reveals the exclusive localization of PKC ε in the steroidogenic cells. The amount of protein used for Western blot was 10 μg/lane. Each lane indicates an individual CL. Panel B shows the semi-quantitative analysis of Western blots to reveal the cellular source of luteal PKC isozymes in day-10 CL ($n = 3$). The *y*-axis shows the ratio of optical density (O.D.) of each luteal PKC isozymes corrected by the detected O.D. for its corresponding actin. Data are presented as the mean ± S.E.M.; values with different letters denote statistically significant differences ($P < 0.05$).

The immunohistological assay of day-10 CL sections revealed that PKC ε was detectable in what appeared to be LLC and SLC (Fig. 3, Panel C). Immunoreactivity in these cells was specific, because it was abolished in all the controls tested (Fig. 3, Panel A and B). Immunoreactivity was not observed in endothelial cells of any of the vascular components examined (Fig. 3).

3.3. Experiment 3

Fig. 4A displays a representative blot obtained with the protein samples isolated from tissue of experiment 3 to examine the cellular redistribution of PKC ε as a function of stimulating the luteal tissue with ET-1 (100nM). In luteal tissue samples treated with MEM-HEPES (Media), PKC ε was detected only in the cytoplasm (Fig. 4A, right panel). In contrast, stimulating the luteal tissue with ET-1 resulted in the detection of PKC ε in the cell membrane fraction (Fig. 4A, left panel). Fig. 4B summarizes the data for PKC

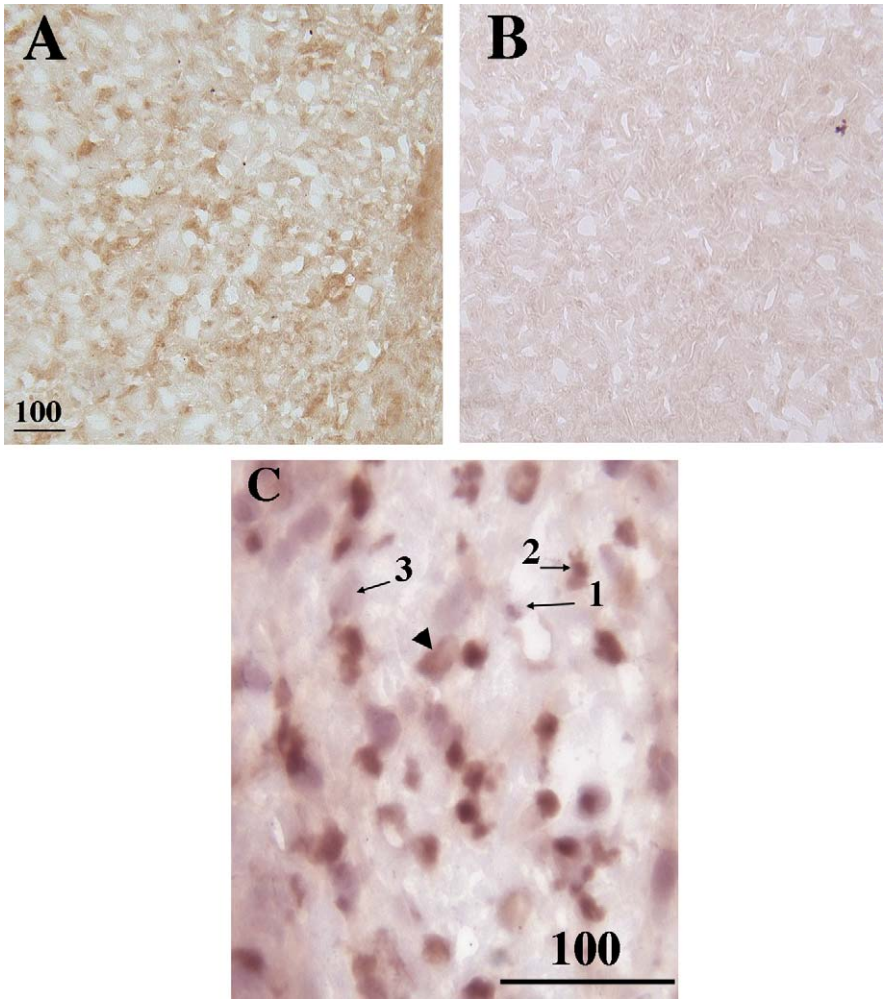

Fig. 3. Immunohistological detection of PKC ε in frozen sections of day-10 CL. Panel A is a lower magnification view of the field shown in panel C. Panel B shows a negative control using a consecutive section to that used in panel A but omitting the primary antibody in the detection protocol. The arrow with the number 1 in panel C indicates the endothelial cell lining the lumen of a blood vessel; observe that this cell lining the lumen of the vessel is not immunopositive. The arrow with the number 2 indicates an immunopositive SLC, while the arrowhead represents an immunopositive LLC. The arrow with the number 3 indicates a LLC without immunoreactivity. Microphotographs shown in panels A and B were taken at the same magnification. The bars in panel A and C represent 100 μm.

redistribution for all the isozymes tested. In spite of the fact that in MEM-HEPES-treated luteal samples (Fig. 4B) PKC α, βI and βII were detected in both the cytoplasmic and cell membrane fractions, ET-1 was able to increase the amount of PKC α and βI detected in the cell membrane fraction ($P < 0.05$). In contrast, no cellular re-distribution of PKC βII was induced by similar ET-1 stimulation (Fig. 4B). In our previous study [7], PKC α was

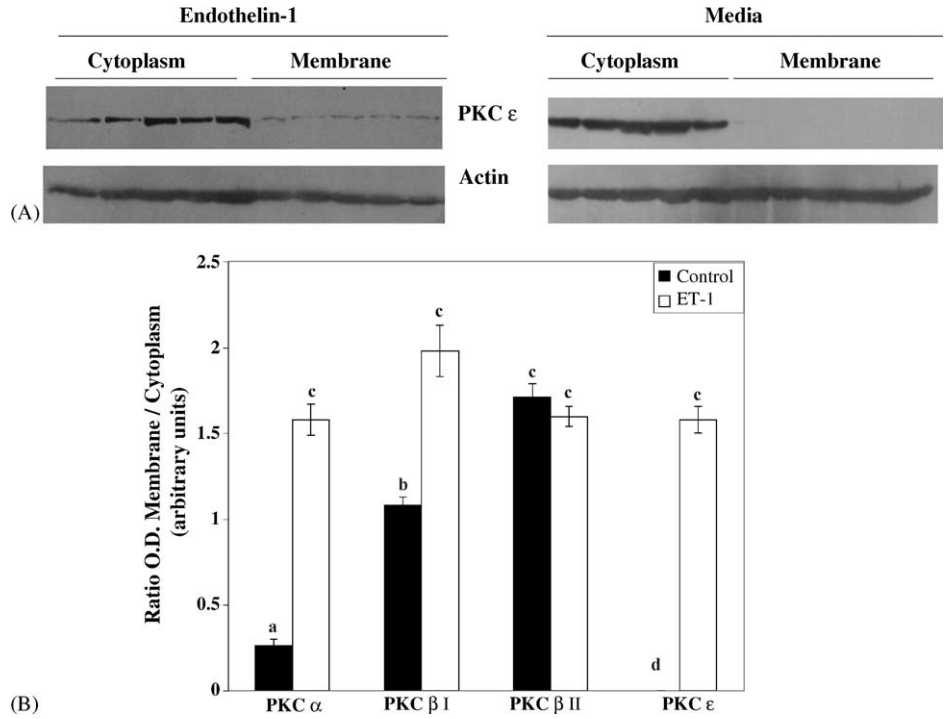

Fig. 4. Panel A shows a representative Western blot demonstrating ET-1 stimulated PKC ε redistribution in day 10 luteal tissue ($n = 5$). Right top panel shows the exclusive cytoplasmic localization of PKC ε when the tissue was incubated with control media, MEM-Hepes. Left top panel demonstrates that a 10 min incubation of the luteal tissue with ET-1 (100 nM) induced the appearance of PKC ε in the membrane fraction. Lower left and right panels show the amount of actin associated with each sample. Each lane indicates individual CL. Amount of protein used for the western blots was 10 μg/lane. Panel B shows the semi-quantitative analysis of ET-1 stimulated PKC redistribution. The *y*-axis represents the actin corrected ratio of the optical density (O.D.) detected for each PKC isozyme in the membrane and cytosolic fractions (M/C). Data are presented as the ±S.E.M.; values with different letters denote statistically significant differences ($P < 0.05$).

detected only in the cytoplasmic fraction of media-treated samples. In the current study, in one of five animals, PKC α was detected in both membrane and cytosolic fraction in media-treated samples. The reason for this discrepancy is unclear, but it is reasonable to assume that it might be due to variability among samples.

3.4. Experiment 4

To analyze the involvement of luteal PKC isozymes in the inhibitory actions of ET-1 on P_4 accumulation, we measured basal and LH-induced P_4 accumulation in the presence and absence of conventional PKC and PKC ε specific inhibitors [22]. The 100 nM concentration of ET-1 used in this study was selected based on a previous study [8].

In day-4 enriched steroidogenic cells ET-1 and LH had no effect on basal P_4 accumulation (Fig. 5A). In contrast, when cells were incubated with the combination of ET-1 and

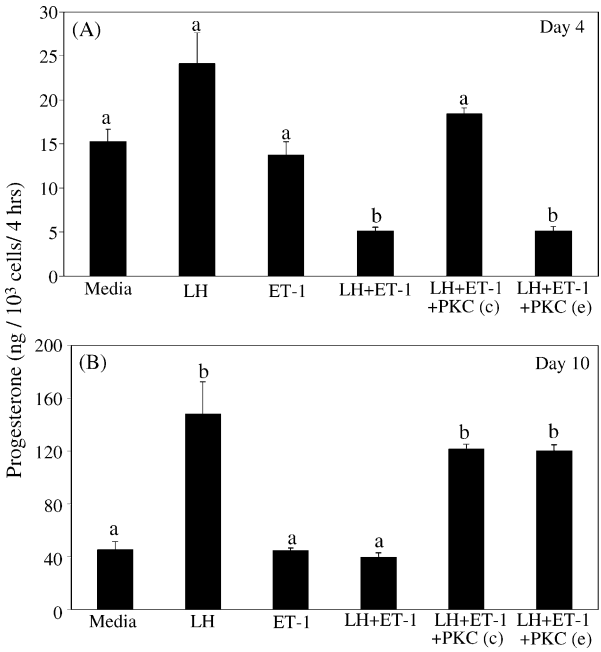

Fig. 5. Effects of conventional PKC [PKC (c)] and PKC ε inhibitors [PKC (e)] on ET-1 (100 nM) actions on P_4 accumulation in cultures of steroidogenic cells collected from (A) Day 4 and (B) Day 10 bovine CL. P_4 accumulation was determined in culture media after 4 h of incubation. Data are presented as the ±S.E.M.; values with different letters denote statistically significant differences ($P < 0.05$).

LH, values for P_4 accumulation were below those observed under basal condition; the conventional PKC inhibitor significantly ($P < 0.05$) antagonized this inhibitory effect (Fig. 5). In contrast, the PKC ε inhibitor did not antagonize this inhibitory effect induced by the combined LH and ET-1 treatment.

Basal P_4 accumulation in cultures of day-10 steroidogenic cells was three times higher than in day-4 cells for all the treatments tested (Fig. 5B). ET-1 had no effect on basal P_4 accumulation. However, LH induced a significant ($P < 0.05$) stimulatory effect in the accumulated P_4 and ET-1 significantly ($P < 0.05$) inhibited this effect of LH. Both conventional as well as ε specific PKC inhibitor were able to negate the inhibitory effect of ET-1 on LH-stimulated P_4 accumulation (Fig. 5B).

4. Discussion

The Western blot and immunohistological data presented here argue strongly in favor of the interpretation that steroidogenic cells constitute the source of PKC ε in the bovine CL. This important observation supports our previous suggestion that this isozyme may play an important role in regulation of P_4 synthesis in the CL of the mid-late phase [10]. We have suggested that a physiological role for PKC ε could be to regulate the ability of $PGF_{2\alpha}$ to induced a rise in $[Ca^{2+}]_i$ capable of inhibiting luteal P4 production. For this to be

feasible, both the $PGF_{2\alpha}$-receptor and PKC ε must be expressed in the same luteal cell type. Furthermore, our immunohistological data strongly indicate that PKC ε is found in both large and small steroidogenic luteal cells. This observation supports the report that the $PGF_{2\alpha}$-induced rise in $[Ca^{2+}]_i$ was decreased in LLC and SLC when their PKC ε was inhibited [10].

As one would expect, there is some cell-cross contamination in any isolated enriched-cell population and consequently, it could be argued that detection of PKC α, βI and βII expression in endothelial cells could be due to contamination with steroidogenic cells. However, considering that PKC α, βII and ε are expressed in similar amounts in day-10 CL [9]; it would appear unlikely that only PKC α, and βII but not PKC ε would be detected if this would be due to contamination of the EC population. Based on this argument, we favor the interpretation that detection of these isozymes in the Western blot with protein isolated from EC is likely due to their being expressed by these cells rather than by SC contamination. Although PKCα, βI and βII were expressed in steroidogenic and endothelial luteal cells, their amounts were significantly less in endothelial than in steroidogenic cells. Whether this difference between these two luteal cell types has any physiological significance is still unclear. Interestingly, Wu and Wiltbank [28] reported that PKC α and β acting on E-box DNA elements specifically increased Cox-2 transcription in LLC. As far as we know, the role for these PKC isozymes in luteal endothelial cells has not been examined previously.

The lower expression of mRNA encoding PKC ε in the early luteal stage (day 1 and day 4) than in the mid-late luteal stage (day 10 and day 17) supports our previous observation of differential expression of PKC ε protein as a function of development [9]. We reported that the amount of protein corresponding to PKC ε was barely detectable in day-4 CL, while it was significantly up-regulated in the day-10 CL [9]. These observations indicate that the lower amount of PKC ε protein in the early CL, at least in part, is due to lower availability of PKC ε mRNA at this developmental stage. This interpretation is supported further by the observation that the amount of total mRNA necessary to amplify a cDNA product corresponding to PKC ε in the day-10 samples was lower than that needed from day-1. However, the regulatory mechanism(s) of PKC ε gene expression during CL development are currently not known.

The potential physiological significance of the differential expression of PKC ε during the development of the CL is that it could participate in the cellular mechanisms rendering the early CL resistant to the antisteroidogenic actions of $PGF_{2\alpha}$. Alternatively, this could simple reflects the immaturity of the cells undergoing luteinization, at this time, this simpler interpretation cannot be ruled out. Importantly, the ability of ET-1, like $PGF_{2\alpha}$ [9], to activate PKC α, βI and especially ε in day-10 steroidogenic cells underlines the importance of PKC ε in luteal regression. Choudhary et al. [22], demonstrated that ET-1 induced increases in $[Ca^{2+}]_i$ in steroidogenic and endothelial luteal cells. The ability of ET-1 to activate the metabolism of phosphoinositides, intracellular calcium and PKC also has been reported in swine granulosal cells [23], rat gonadotropes [29] and many other tissues [30,31]. All these observations further support the idea that ET-1, like $PGF_{2\alpha}$ utilizes, at least in part, the PLC effector system with PKC and calcium as intracellular mediators.

We have proposed that ET-1 appears to be a tonic inhibitor of luteal P_4 production [22] rather than a mediator of $PGF_{2\alpha}$ actions as suggested by other investigators [14–16]. We previously demonstrated that ET-1 treatment of early CL cells in vitro inhibited basal and LH-stimulated P_4 accumulation [22] on both. However, in this study we did not observe

any effect of LH nor ET-1 over basal P_4 accumulation. This discrepancy is most likely due to the permeabilization protocol used in this study. This interpretation is further supported by the observations in a previous study when the same procedure was performed, no effect of LH was observed in day-4 luteal steroidogenic cells. However, in the present study, an inhibitory interaction between ET-1 and LH was observed on the amount of basal P_4 (Fig. 5). This interesting interaction might be due to a combined effect of both ET-1 and LH in increasing $[Ca^{2+}]_i$, which would in turn possibly activate conventional PKC. This interpretation is supported by the observation that the conventional PKC inhibitor reversed this negative interaction, bringing P_4 accumulation values back to that observed under basal condition. The ability of ET-1 to exert this action on day-4 luteal tissue supports the report that day-4 CL, although less sensitive than day-10 CL, are responsive to ET-1 (20). In this context, in vivo ET-1 availability in its specific luteal target cells in the early CL would be critical in determining tissue responsiveness. If, indeed ET-1 is available in the early CL, our studies indicate that regulation of P_4 accumulation at this stage is most likely dependent on conventional PKC isozymes and independent of PKC ε.

In contrast and supporting previous studies [9,22], in day-10 luteal cells LH stimulated P_4 accumulation over basal values and ET-1 inhibited this effect of LH. The inhibitory effect of ET-1 on LH-stimulated P_4 accumulation was antagonized by both conventional and PKC ε specific inhibitors. This underlines that during this developmental stage, due to an increased PKC ε expression and availability of two demonstrated ligands ($PGF_{2\alpha}$ and ET-1) with the capacity to activate this isozyme, PKC ε becomes at least as potent as conventional PKC isozymes in mediating inhibitory actions on P_4 accumulation. However, whether the actions of PKC ε are synergistic or additive to those of conventional PKC isozymes needs further investigation. We propose that adding availability of PKC ε to the already available action of conventional PKC isozymes during the mid luteal phase constitutes an important factor that might shift the balance towards luteal regression.

In summary, presence of PKC ε exclusively in steroidogenic cells, with higher availability of PKC ε mRNA and protein [9] in the mid-late CL, along with its potential inhibitory role on P_4 accumulation in the mid-late CL [10] underline the importance of this isozyme in luteal regression. The differential expression of PKC ε may be one of several key factors responsible for the sensitivity of the CL to luteal regression. Moreover, the tonic inhibition of P_4 accumulation by ET-1 [22] in the early CL may be mediated via conventional PKC isozymes.

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