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Prostaglandin $F_{2\alpha}$-Activated Protein Kinase Cα Phosphorylates Myristoylated Alanine-Rich C Kinase Substrate Protein in Bovine Luteal Cells

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Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced secretion of oxytocin by the bovine corpus luteum involves the phosphorylation of a unique protein kinase C (PKC) substrate, myristoylated alanine-rich C kinase substrate (MARCKS) protein. This study was conducted to determine the specific PKC isoform engaged in phosphorylation of MARCKS protein in bovine luteal cells. In experiment 1, dispersed luteal cells recovered from the corpus luteum on d 8 of the estrous cycle were preincubated with [^{32}P] orthophosphate and then exposed to $PGF_{2\alpha}$ alone or in combination with PKC inhibitors. Autoradiography and densitometry of Western blots revealed that MARCKS protein was phosphorylated by a conventional PKC (cPKC) isoform. Experiment 2 was conducted to identify the specific cPKC isoform that phosphorylates MARCKS protein in luteal cells. Corpora lutea were removed from control and $PGF_{2\alpha}$-treated heifers on d 8 of the cycle, and PKC isoforms associated with membrane and cytosolic fractions were determined. Treatment with $PGF_{2\alpha}$ increased membrane concentrations of PKCα within 5 min after treatment ($p < 0.005$). Collectively, these data suggest that phosphorylation of MARCKS protein coinciding with oxytocin secretion is mediated by PKCα.

Key Words: Protein kinase C; prostaglandin $F_{2\alpha}$; MARCKS protein; corpus luteum; bovine.

Introduction

The ruminant corpus luteum consists of small and large steroidogenic cells *(1,2)*. Both cell types produce progesterone; however, only large luteal cells synthesize and secrete oxytocin *(3)*. It is known that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) acts on the luteal cells to stimulate a phosphoinositide cascade with a consequent increase in intracellular calcium and activation of protein kinase C (PKC) *(4)*. Activation of PKC coincides with exocytosis of oxytocin from luteal cells *(5)*, suggesting that phosphorylation of proteins is a requisite step in this biologic phenomenon. One such protein is a myristoylated alanine-rich C kinase substrate (MARCKS) protein whose function in the unphosphorylated state is to crosslink the actin filaments that constitute the cytoskeletal actin cortex of the cell *(6,7)*. Phosphorylation of MARCKS by PKC reduces its membrane binding and actin crosslinking activity *(6)* and results in its translocation from membrane to cytoplasm with a consequential disassembly of the actin cortex *(8,9)*. In the bovine corpus luteum, MARCKS mRNA and protein exist throughout the estrous cycle *(10)*. Exposure of luteal cells to $PGF_{2\alpha}$ under in vitro or in vivo conditions results in phosphorylation of MARCKS, and its translocation from the plasma membrane to cytoplasm *(11)*. $PGF_{2\alpha}$-induced translocation of MARCKS in luteal cells is correlated timewise with activation of PKC and exocytosis of oxytocin *(5,11)*. Previous evidence suggests that the bovine corpus luteum contains several PKC isoforms including PKCα, PKCβI, PKCβII, and PKCε *(12,13)*. However, which isoform (or isoforms) of PKC undergoes $PGF_{2\alpha}$-induced activation and is responsible for the phosphorylation of MARCKS remains to be determined. The purpose of the present study was to determine the specific PKC isoform (or isoforms) employed for phosphorylation of MARCKS in the bovine corpus luteum.

Results

Experiment 1

Results of this experiment are depicted in Figs. 1 and 2 and consist of an autoradiograph (Figs. 1A and 2A) of a Western blot (Figs. 1B and 2B) denoting a single band of MARCKS protein (87 kDa) and graphic depiction of the data (Figs. 1C and 2C) expressed as the ratio of densitometric units of [^{32}P]-phosphorylated MARCKS to those of the transferred protein.

To determine the particular PKC isoform (or isoforms) responsible for MARCKS phosphorylation in the bovine corpus luteum, a plasma membrane–permeable myristoylated inhibitor peptide, which is a pseudosubstrate for PKCα, PKCβ, and PKCγ isoforms, was added to the incubation medium containing luteal cells. Similar to data reported previously *(11)*, treatment of dispersed luteal cells with $PGF_{2\alpha}$

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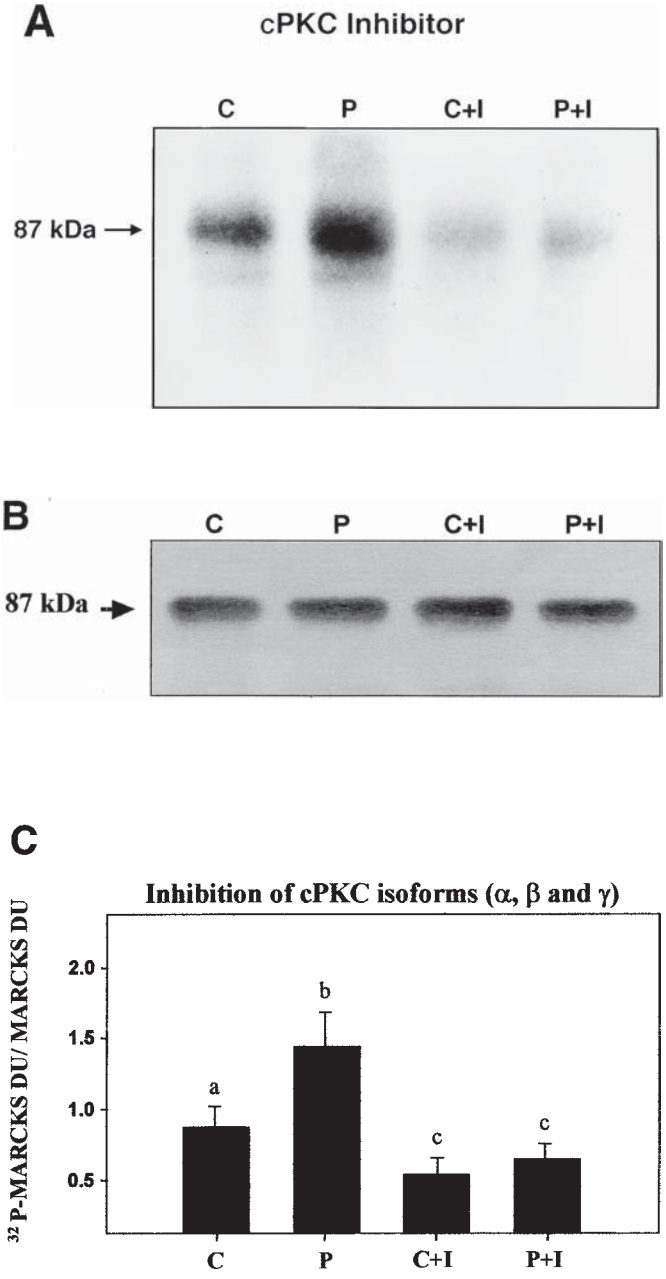

Fig. 1. (**A**) Effects of myristoylated cPKC inhibitor on phosphorylation of MARCKS determined by autoradiography of Western blots. Luteal cells were incubated with [^{32}P]-orthophosphate in the absence or presence of cPKC inhibitor peptide. Equal quantities of cellular protein samples were subjected to 7.5% SDS-PAGE and then transferred to nylon membranes to obtain autoradiographs and Western blots. (**B**) The Western blot of the membrane used to obtain autoradiographs in (A) displays one single band and an equal amount of protein in each lane. The arrow indicates phosphorylated MARCKS protein (87 kDa). (**C**) Changes in total phosphorylation of MARCKS in luteal cells exposed to treatments are represented as the ratios of densitometric units (DU) of phosphorylated MARCKS in (A) to that of MARCKS protein in the corresponding lane in (B). C, Control (35 μL of ethanol); P, $PGF_{2\alpha}$ (56 n*M* cloprostenol); C+I, control and myristoylated cPKC inhibitor (100 μ*M*); P+I, $PGF_{2\alpha}$ and myristoylated cPKC inhibitor. Data are the means of four animals and are represented as arbitrary DU. Means (±SE) with different superscript letters differ ($p < 0.05$).

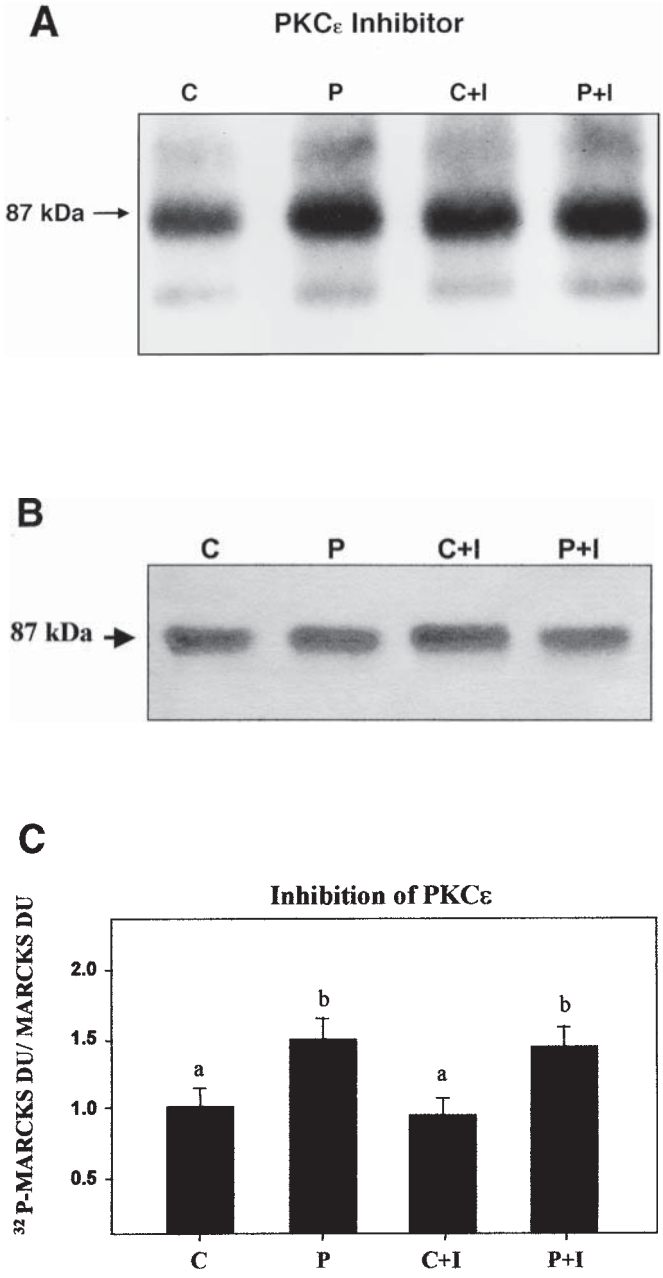

Fig. 2. (**A**) Effects of PKCε inhibitor on phosphorylation of MARCKS determined by autoradiography of Western blots. Luteal cells were incubated with [^{32}P]-orthophosphate in the absence or presence of PKCε inhibitor peptide. Equal quantities of cellular protein samples were subjected to 7.5% SDS-PAGE and then transferred to nylon membranes to obtain autoradiographs and Western blots. (**B**) The Western blot of the membrane used to obtain autoradiographs in (A) displays one single band and an equal amount of protein in each lane. The arrow indicates phosphorylated MARCKS protein (87 kDa). (**C**) Changes in total phosphorylation of MARCKS in luteal cells exposed to treatments represented as the ratios of densitometric units (DU) of phosphorylated MARCKS in (A) to that of MARCKS protein in the corresponding lane in (B). C, Control (35 μL of ethanol); P, $PGF_{2\alpha}$ (56 n*M* cloprostenol); C+I, control and PKCε inhibitor (150 μ*M*); P+I, $PGF_{2\alpha}$ and PKCε inhibitor. Data are the means of four animals and are represented as arbitrary DU. Means (±SE) with different superscript letters differ ($p < 0.05$).

significantly increased phosphorylation of MARCKS protein relative to controls (Fig. 1A,C). The addition of the myristoylated inhibitor peptide to the medium significantly suppressed phosphorylation of MARCKS protein in the absence or presence of $PGF_{2\alpha}$ compared with that in control cells or those exposed to only $PGF_{2\alpha}$ (Fig. 1A,C; $p < 0.05$).

Involvement of PKCε in MARCKS protein phosphorylation was also tested in this experiment by using a PKCε inhibitor peptide whose plasma membrane permeability was facilitated by the addition of saponin (0.005%) to the incubation medium. Incubation of luteal cells with PKCε inhibitor peptide alone or in combination with $PGF_{2\alpha}$ failed to prevent phosphorylation of MARCKS protein. Phosphorylation of MARCKS protein in cells treated with $PGF_{2\alpha}$ and $PGF_{2\alpha}$ plus inhibitor was greater than in respective control cells (Fig. 2A,C; $p < 0.05$). Phosphorylation of MARCKS protein in control cells incubated alone or with PKCε inhibitor peptide did not differ ($p > 0.05$).

Experiment 2

Depending on cell type, when the PKC activators (Ca^{2+} and diacylglycerol [DAG]) are present in the intracellular milieu, the majority of PKC isoforms including PKCα, PKCβI, PKCβII, and PKCε translocate from cytoplasm to the plasma membrane in order to become active *(14,15)*. Therefore, the purpose of experiment 2 was to determine the subcellular distribution of PKC isoforms upon $PGF_{2\alpha}$ injection of heifers. Western blots conducted for this purpose revealed that treatment with $PGF_{2\alpha}$ resulted in a visible increase in the quantity of PKCα associated with the membrane fraction compared with the quantity of this isozyme present in the cytosol (Fig. 3). In both control and $PGF_{2\alpha}$-treated animals, with the exception of PKCα, there were significant quantities of each of the other isoforms present in the cytosol and visibly meager quantities in the membrane. After calculating the ratios between densitometric unit values of membrane and cytosol fractions of individual isoforms (Fig. 3C), translocation of PKCα to the membrane of $PGF_{2\alpha}$-treated cells was determined to be approximately sixfold greater ($p < 0.005$), while there was no significant difference in other isoform translocations.

Although there was more membrane-associated PKCε than PKCβI or PKCβII, there was no increase in membrane association of any of these three isoforms with $PGF_{2\alpha}$ treatment.

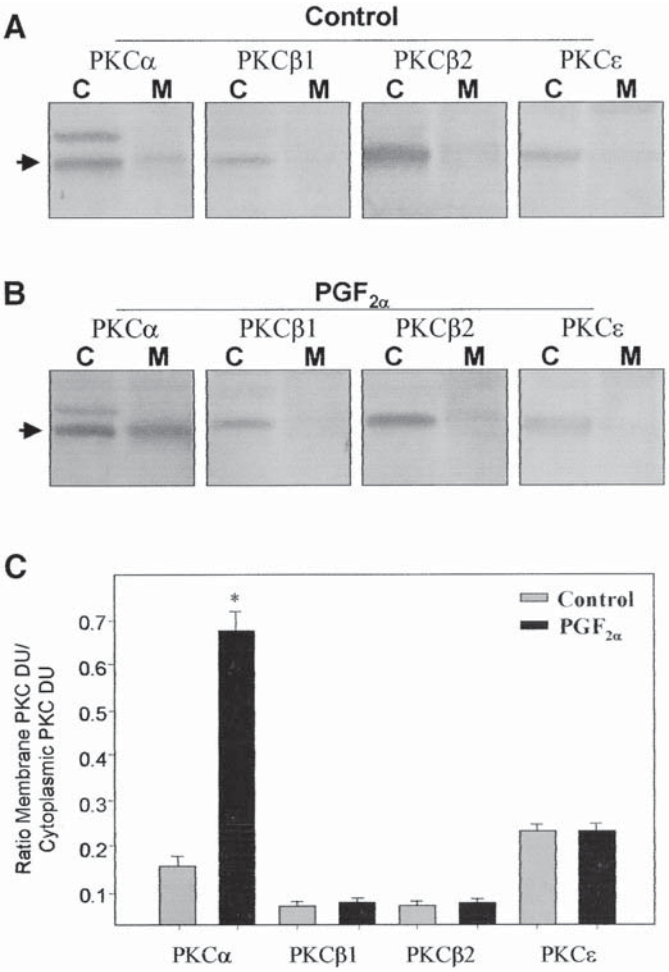

Fig. 3. The upper panels depict representative Western blots of the intracellular distribution of four PKC isoforms in saline (**A**) and $PGF_{2\alpha}$-treated (**B**) animals. For comparisons, each individual PKC isoform was aligned based on PKCα (76 kDa) indicated by the arrows. Molecular weights for the remaining isoforms are as follows: PKCβI (76.8 kDa), PKCβII (77 kDa), and PKCε (83 kDa). Four equal amounts (200 μg/lane) of the same protein sample were loaded from both cytosol (C) and membrane (M) fractions. Samples were applied to 7.5% SDS-PAGE and then transferred to nylon membranes. Each lane containing either the cytosolic or membrane fraction was strip cut and subjected to Western blotting for one of the four PKC isoforms. (**C**) Effects of $PGF_{2\alpha}$ stimulation on subcellular distribution of PKC isoforms as represented in (A). These data are expressed as the ratio of densitometric units (DU) of membrane PKC to that of cytosolic PKC as shown in (A) and (B) and are the means of four animals/group. The mean with an asterisk differs from the control ($p < 0.005$). The upper band visible in the PKCα lane (relatively less in other lanes) may be caused by the nonspecific binding of the polyclonal antibody.

Discussion

We previously reported that $PGF_{2\alpha}$ and 12-O-tetradecanoylphorbol-13-acetate (a potent PKC activator) stimulated phosphorylation of MARCKS by activating PKC in bovine luteal cells in vivo *(11)*. Because the bovine corpus luteum contains conventional PKC (cPKC) isoforms (α, βI, and βII) and novel PKCε (nPKCε) *(12,13)*, which specific PKC isoform (or isoforms) is responsible for phosphorylation of MARCKS remains to be elucidated. In the present study, we used two strategies to investigate the specific PKC isoforms responsible for $PGF_{2\alpha}$-induced phosphorylation of MARCKS protein. In the first approach, specific PKC inhibitors were used to detect the changes in phosphorylation of MARCKS in response to $PGF_{2\alpha}$ treatment. All PKC

isoforms, except PKCμ, possess a pseudosubstrate domain that resembles their substrate *(16,17)*. It is proposed that the pseudosubstrate domain interacts with the substrate-binding site in the catalytic domain of PKC and maintains the kinase in the inactive state *(16)*. The presence of PKC activators such as DAG or phorbol esters interrupts the interaction between pseudosubstrate and substrate-binding domain, resulting in substrate binding and subsequent phosphorylation of substrate. The myristoylated PKC inhibitor peptide used in experiment 1 has been found to inhibit phosphorylation of MARCKS protein in human fibroblasts by blocking the activation of cPKC isoforms *(18)*. Incubation of luteal cells with this peptide in experiment 1 resulted in significant reduction in phosphorylation of MARCKS, suggesting that one or more of the cPKC isoforms phosphorylates the MARCKS protein.

The other isoform examined was PKCε, a member of the nPKC family. The function of this isozyme was studied because PKCε has been detected in both cytosol and membrane fractions of bovine corpus luteum, suggesting its potential to phosphorylate MARCKS *(12)*. An inhibitor of PKCε has been reported to be selectively inhibitory of translocation of PKCε in transiently permeabilized neonatal rat cardiomyocytes *(19)*. In experiment 1, incubation of transiently permeabilized luteal cells with PKCε inhibitor had no effect on either basal or $PGF_{2\alpha}$-stimulated phosphorylation of MARCKS.

The second approach used to define the PKC isozyme that phosphorylates MARCKS was based on the fact that the majority of PKC isoforms (including PKCα, PKCβI, PKCβII, and PKCε) translocate from cytoplasm to plasma membrane on stimulation *(14,15)*. Previous research in our laboratory demonstrated that iv injection of $PGF_{2\alpha}$ into heifers caused a significant increase in luteal membrane PKC activity within 5 min, which is concomitant with luteal secretion of oxytocin *(5)*. Therefore, subcellular distribution of PKC isoforms 5 min after $PGF_{2\alpha}$ injection of heifers was determined. Results of Western blot analysis using affinity purified antibodies specific for the various isoforms of PKC revealed that only quantities of PKCα associated with the plasma membrane increased (approximately sixfold) compared with that of the saline-treated animals, which suggests that $PGF_{2\alpha}$-induced phosphorylation of MARCKS is mediated by PKCα. This result is in agreement with previous studies reporting that the release of noradrenaline is enhanced by phorbol ester–induced migration of PKCα to the plasma membrane but not PKCε or PKCζ in SH-SY5Y human neuroblastoma cells *(20,21)*. On the other hand, using bovine luteal slices, Browning et al. *(22)* found that only PKCβII transiently translocated from cytoplasm to the plasma membrane fraction. The conflicting results between our study and theirs *(22)* may be owing to the experimental models and methods. In contrast to their findings, our data are based on in vivo treatment of animals.

Phosphorylation of MARCKS disrupts its ability to bind and crosslink actin filaments resulting in rearrangements of the cytoskeleton that are believed to lead to exocytosis *(7,9,23)*. The evidence supporting this phenomenon comes from various studies including phorbol ester–induced prolactin secretion from GH_4C_1 cells *(24)*, arginine vasopressin-induced release of adrenocorticotropic hormone from pituitary cells *(25)*, pepsinogen release from gastric chief cells *(26)*, and glucose-induced insulin secretion *(27)*. Recent studies have indicated that MARCKS is efficiently phosphorylated by cPKC and nPKC isoforms but not by atypical PKC isoforms *(28,29)*. Although PKCα, PKCβI, PKCβII, PKCδ, PKCε, and PKCζ are distributed ubiquitously in tissue, it appears that the function of each individual PKC isoform has a unique cell-tissue specificity *(30)*. This may, in part, reflect their requirements for activation. Phospholipid and DAG are required for activation of nPKC isoforms (δ, ε, θ, η, and μ) while phospholipid, DAG, or phorbol ester and Ca^{2+} are required for activation of cPKC isoforms. On the other hand, the aPKC isoforms (ζ and λ) require only phospholipid *(31–33)*. Our previous study has indicated that the addition of A23187, a Ca^{2+} ionophore, to incubation media increased phosphorylation of MARCKS in bovine luteal cells, suggesting that at least one of the cPKC isoforms was a potential regulator of MARCKS phosphorylation *(11)*.

In conclusion, PKCα appears to be the major, if not the only, kinase to phosphorylate MARCKS in the bovine corpus luteum. Our study sheds more light on $PGF_{2\alpha}$-induced activation of the PKC system and aspects of MARCKS phosphorylation that are involved in exocytosis of oxytocin by the large luteal cells.

Materials and Methods

Animals

Twelve beef heifers were checked twice daily for behavioral estrus (d 0), using a vasectomized bull. The corpus luteum was collected on d 8 of the cycle per vaginum under lidocaine (2%)-induced caudal epidural anesthesia *(5)*. On removal, the corpus luteum to be utilized in experiments involving phosphorylation of proteins was transported to the laboratory in a phosphate-deficient medium (Dulbecco's modified Eagle's medium [DMEM]; Gibco-BRL, Grand Island, NY) containing gentamicin (30 μg/mL) (Gibco-BRL) at 4°C. The corpus luteum utilized in experiments involving Western immunoblotting was transported in 1X F-12 Nutrient (Ham) Mixture (Gibco-BRL) containing gentamicin at 4°C. All experimental procedures were performed in accordance with the institutional guidelines for the care and use of animals.

[^{32}P]-Orthophosphate was purchased from Dupont (Boston, MA). All chemicals were purchased from Sigma (St. Louis, MO), Bio-Rad (Hercules, CA), or Boehringer Mannheim (Indianapolis, IN) unless otherwise reported.

Experiment 1:
Altered In Vitro Phosphorylation of MARCKS by cPKC and PKCε Inhibitors

Experiment 1 was conducted to determine whether a cPKC isoform or PKCε is responsible for $PGF_{2\alpha}$-stimulated phosphorylation of luteal MARCKS protein in vitro. Dispersion of luteal cells and other aspects of in vitro phosphorylation were described previously *(11)*. Briefly, minced luteal tissue was dissociated with collagenase (3000 U/g of tissue) in 25 mL of DMEM containing DNase I (1.4 U/mL). After determining the percentage of viable cells (range: 73–85%), aliquots of 1×10^7 live luteal cells were placed into eight flasks containing 2 mL of phosphate-deficient DMEM, then incubated for 1.5 h at 37°C under an atmosphere of 95% O_2-5% CO_2 in order to deplete endogenous phosphate. [^{32}P]-Orthophosphate (100 μCi/mL) and PKC inhibitors (described subsequently) were then added to all flasks, and incubation was continued for another 1.5 h. The experiment consisted of two groups, group 1 and group 2, each containing four flasks of dispersed luteal cells. In group 1, a myristoylated PKC inhibitor (100 μ*M*) (Promega, Madison, WI) was used to determine the effects of inhibition of cPKC isoforms. This peptide contains the pseudosubstrate region of PKCα and PKCβ and differs by one amino acid residue from PKCγ. The addition of myristic acid to this peptide enhances its permeability of the plasma membrane allowing the inhibition of cPKC isoforms. At the end of the 1.5-h phosphorylation incubation, the cells in the four flasks were treated with vehicle (35 μL of ethanol) or $PGF_{2\alpha}$ (56 n*M* cloprostenol; Bayer, Shawnee Mission, KS) to achieve the following treatment conditions during an additional 20-min incubation: controls, $PGF_{2\alpha}$ myristoylated PKC inhibitor (MPI) (100 μ*M*), and $PGF_{2\alpha}$ + MPI.

In group 2, the effects of the PKCε inhibitor peptide (150 μ*M*) (Calbiochem, La Jolla, CA), which is known to inhibit activation of PKCε, were tested. Luteal cells in this group were permeablized by the addition of saponin (0.005%) to the incubation medium for the final 20 min of the phosphorylation incubation to enable PKCε inhibitor peptide to cross the cell membrane. This concentration of saponin has been used previously without exhibiting any detrimental effects on cell viability *(18)*. Four flasks were assigned as already described and at the end of a 1.5-h incubation received vehicle (35 μL of ethanol) or $PGF_{2\alpha}$ (56 n*M*) to result in the following treatment combinations for an additional 20-min incubation: controls, $PGF_{2\alpha}$, PKCε inhibitor (PεI) (150 μ*M*), and $PGF_{2\alpha}$ + PεI.

Incubation of cells in groups 1 and 2 was terminated by placing the flasks on ice, and cells were homogenized with a glass Dounce tissue grinder (Wheaton, Millville, NJ) in 1 mL of buffer A (50 m*M* Tris-HCl, pH 8.3; 5 m*M* EDTA; 0.15 *M* NaCl) containing enzyme inhibitors (5 μ*M* microcystin, 1 μ*M* calpeptin, 1X protease inhibitor cocktail set I; Calbiochem). Cell homogenates were subjected to a low-speed centrifugation (1000*g*) for 10 min to remove the cell nuclei. The supernatant containing total proteins was boiled, and heat-stable proteins including MARCKS were separated by medium-speed centrifugation (15,000*g*) for 10 min. Separating the heat-stable proteins decreases the number of proteins subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), thus reducing the background binding in Western blots and decreasing the number of phosphorylated proteins in the autoradiographs. Aliquots of proteins (100 μg) from this final supernatant from both control and treated cells were subjected to 7.5% SDS-PAGE (10 mA, 200 V, 16 h) and then transferred to a nylon membrane (210 mA, 200 V, 5 h) that was then removed, covered with plastic wrap, and exposed to a sensitive developing film (Hyperfilm; Amersham Pharmacia Biotech, Piscataway, NJ) for autoradiography. After obtaining an adequate autoradiographic image, nylon membranes were processed for Western blotting *(11)* to confirm the precise location of phosphorylated MARCKS on the autoradiograph and to perform densitometric quantification of the transferred protein. For Western blotting, a mouse monoclonal MARCKS antibody (1:500 in 2.5% nonfat dry milk, 1 h at 25°C) (Upstate Biotechnology, Lake Placid, NY) and goat antimouse IgG–alkaline phosphatase secondary antibody (1:2000 in 2.5% nonfat dry milk, 30 min at 25°C) (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Data are expressed as the ratio of densitometric units of [^{32}P]-phosphorylated MARCKS to those of the transferred MARCKS protein.

Experiment 2:
Subcellular Translocation of PKC Isoforms in Response to $PGF_{2\alpha}$ Treatment

Experiment 2 was performed to identify the specific cPKC isoform (or isoforms) in experiment 1 that was responsible for phosphorylation of MARCKS. Eight heifers were assigned randomly to control ($n = 4$) and treatment ($n = 4$) groups. Colpotomy was performed as described earlier, but the corpus luteum was not removed until 5 min after initiating iv treatments of saline (2 mL) or $PGF_{2\alpha}$ (500 μg of cloprostenol/2 mL). After removal, the corpus luteum was homogenized with buffer A (1 mL/g of tissue) using a Tekmar Tissuemizer (Tekmar, Cincinnati, OH). The homogenate of the corpus luteum of each heifer was subjected to a series of centrifugations to obtain cytosolic and membrane fractions *(11)*. After determining protein concentrations of these fractions, four replicates (200 μg/lane × 4) of cytosolic and membrane proteins of each corpus luteum were applied to 7.5% SDS-PAGE and transferred to a nylon membrane. Each lane on the nylon membrane was strip cut representing a single lane of the same sample. The nylon membrane background was blocked with 5% nonfat dry milk. Each strip was then immunoblotted with one of the following: polyclonal rabbit-PKCα, rabbit-PKCβI, rabbit-PKCβII, or rabbit-

PKCε antibodies (1 µg/mL of each affinity-purified antibody; Santa Cruz Biotechnology). The primary antibody-protein bindings were detected by probing with the polyclonal goat antirabbit secondary antibody (1:2000) conjugated with alkaline phosphatase and visualized by incubating with alkaline phosphatase substrate *(11)*. Densitometry readings were performed on related bands of PKC isoforms from Western blots. The data are presented as the ratio of arbitrary densitometric units of membrane PKC to cytosolic PKC.

Statistical Analyses

The data obtained from experiment 1 were analyzed by analysis of variance (ANOVA) for an experiment of complete randomized block design. The differences among means in experiment 1 were tested for significance using the Fisher's protected least significant difference test. Differences among membrane to cytosol ratios of four PKC isoforms in corpus luteum of control and $PGF_{2\alpha}$-treated animals in experiment 2 were tested for significance using ANOVA.

Acknowledgments

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