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Myristoylated Alanine-Rich C Kinase Substrate Protein and mRNA in Bovine Corpus Luteum During the Estrous Cycle

Shelby Filley, Sara Supancic, Ugur Salli, Kyle Orwig, and Fredrick Stormshak

Departments of Biochemistry/Biophysics and Animal Sciences, Oregon State University, Corvallis, OR

The bovine corpus luteum contains a myristoylated alanine-rich C kinase substrate (MARCKS) protein known to crosslink actin filaments in the cytoskeletal cortex associated with the plasma membrane. We conducted experiments to determine whether concentrations of MARCKS mRNA and protein in the bovine corpus luteum varied during the estrous cycle. Using Northern blots probed with a MARCKS cDNA, we found that luteal concentrations of MARCKS mRNA were greatest on d 4, 8, and 12 and markedly reduced on d 16 of the cycle ($p < 0.08$). Similarly, Western blot analysis of luteal proteins revealed that concentrations of MARCKS protein were greatest on d 8 and least on d 16 of the cycle ($p < 0.01$). Exposure of slices from a d 8 corpus luteum to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) during a 10-min incubation in the presence of [^{32}P]-orthophosphate resulted in enhanced phosphorylation of MARCKS in membrane and cytosolic fractions compared to that of controls. We therefore concluded that expression of the luteal MARCKS protein gene may be regulated and that $PGF_{2\alpha}$-induced phosphorylation of this protein is attributable to activation of protein kinase C.

Key Words: MARCKS protein; corpus luteum; estrous cycle; bovine.

Introduction

The corpus luteum is most generally associated only with the production of progesterone in mammalian females. However, in some species, namely ruminants, this endocrine gland also produces significant quantities of oxytocin during certain stages of the estrous cycle *(1,2)*. It has been proposed that luteal oxytocin and uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are functionally interrelated via a positive feedback loop with the oxytocin-induced pulsatile secretion of $PGF_{2\alpha}$ ultimately causing luteal regression in these species *(3)*. This is supported in part by the observation that the administration of $PGF_{2\alpha}$ to ewes and cows during the cycle can stimulate immediate secretion of luteal oxytocin *(4,5)*.

$PGF_{2\alpha}$-induced secretion of oxytocin involves the ability of this eicosanoid to trigger a phosphoinositide cascade in large luteal cells with resultant activation of protein kinase C (PKC) and phosphorylation of a number of proteins *(5–7)*. A consequence of cell stimulation by $PGF_{2\alpha}$ is rapid movement of transport vesicles containing oxytocin-neurophysin granules to the plasma membrane and exocytosis *(8)*. Exocytosis of oxytocin-neurophysin appears to require disassembly of an actin cortex that is in close apposition with the plasma membrane and that otherwise impedes this cellular process. The cortex is composed of F-actin filaments that are crosslinked by a number of proteins, one of which is a myristoylated alanine-rich C kinase substrate (MARCKS) protein *(9,10)*. Phosphorylation of MARCKS by PKC has been shown to cause it to translocate from the membrane location to the cytoplasm, thus promoting disassembly of the actin cortex and facilitating exocytosis *(11)*. The MARCKS protein has been identified as being present in the bovine corpus luteum *(2)*; however, it is not known to what extent luteal concentrations of MARCKS mRNA change during the estrous cycle and whether these changes, if any, result in similar fluctuations in MARCKS protein.

The present experiments were conducted to determine whether luteal concentrations of MARCKS mRNA and protein are altered during the course of the bovine estrous cycle. As a corollary objective, a preliminary attempt was made to ascertain whether in vitro stimulation of luteal tissue with $PGF_{2\alpha}$ would result in MARCKS phosphorylation.

Results

MARCKS mRNA was greatest on days 4, 8, and 12 and lowest on d 16 of the cycle ($p < 0.08$; Fig. 1A,B). No alternate forms of MARCKS mRNA were detected. The MARCKS protein migrated on sodium dodecyl sulfate (SDS)-polyacrylamide gels as though it had a molecular mass equivalent to 87 kDa (Fig. 2A). As observed from the Western blot, the MARCKS protein was present in the bovine corpus luteum on each of d 8, 12, and 16 of the

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Author to whom all correspondence and reprint requests should be addressed: Dr. Fredrick Stormshak, Department of Animal Sciences, Oregon State University, Corvallis, OR 97331. E-mail: fred.stormshak@orst.edu

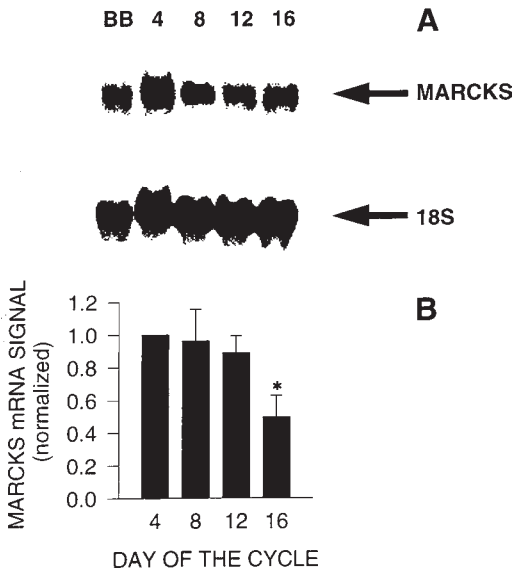

Fig. 1. Northern blot of MARCKS protein mRNA in bovine corpora lutea during d 4, 8, 12, and 16 of the estrous cycle **(A)** and mean (±SE) density of the MARCKS mRNA signal normalized to d 4 MARCKS mRNA **(B)**. Bovine brain (BB) was included as a positive control. 18S rRNA was probed to determine equality of loading of the sample. Quantitation was performed using volume quantitation of equal areas for a specific signal. Background was corrected for by subtraction of the local average around the area quantitated (*see above* 16). *Mean (±SE) luteal concentration of MARCKS mRNA on d 16 differs from those of other days ($p < 0.08$).

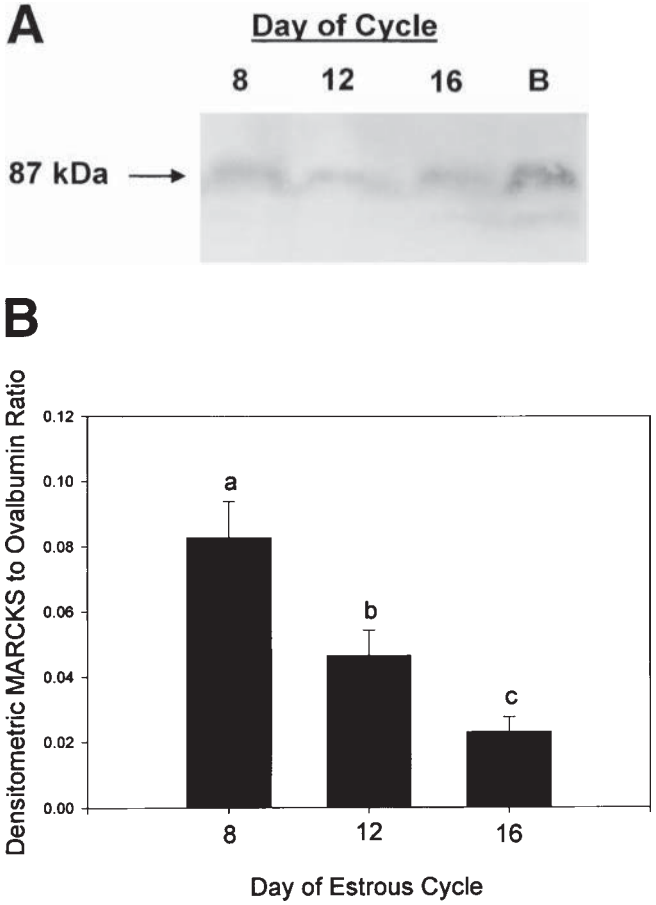

Fig. 2. Western blot of MARCKS protein in bovine corpora lutea on d 8, 12, and 16 of the estrous cycle **(A)** and densitometric representation of protein levels expressed as mean (±SE) MARCKS to ovalbumin ratio **(B)**. Bovine brain (B) was included as a positive control. Means with different superscript letters differ ($p < 0.01$).

cycle. Changes in luteal concentrations of MARCKS protein during the estrous cycle were similar to those of the mRNA. Luteal concentration of MARCKS protein was maximal on d 8 of the cycle and steadily declined to lowest levels by d 16 ($p < 0.01$; Fig. 2B).

Incubation of luteal slices with [^{32}P]-orthophosphate for 10 min resulted in the presence of phosphorylated MARCKS protein in both the cytosolic and membrane fractions of control and treated tissue (Fig. 3A). Compared to respective controls, $PGF_{2\alpha}$ stimulated phosphorylation of MARCKS protein present in the cytosolic and membrane fractions of luteal cells. The Western blot (*see* Fig. 3B) depicts the equality of protein loaded unto the gel for each of the subcellular fractions of control and treated tissue. The data expressed as the ratio of [^{32}P]-labeled MARCKS to transferred protein (Fig. 4) suggest that the phosphorylated MARCKS associated with the membrane and cytosolic fractions of tissue exposed to $PGF_{2\alpha}$ was greater than that of respective controls.

Discussion

The results of our studies indicate that MARCKS mRNA and protein are present in the bovine corpus luteum throughout the estrous cycle. Concentrations of mRNA and protein were greatest through the early part of the midluteal phase of the cycle and were markedly reduced by d 16. The patterns of change in luteal MARCKS mRNA and protein during the cycle are generally similar but a reduction in protein concentration was already detectable by d 12. It is a noteworthy that the luteal expression of the MARCKS gene appears to be congruent with the functional activity of the corpus luteum, i.e., the production of progesterone and oxytocin. For example, luteal concentrations of oxytocin are maximal by around d 8 or 9 of the cycle and decline thereafter to low levels on d 16 *(12)*. Similarly, luteal production of progesterone in the cow is maximal during the midluteal phase of the cycle and then decreases thereafter *(13)*.

The promoter region of the MARCKS gene lacks a TATA box and exhibits characteristics closely resembling those found primarily in "housekeeping genes" *(14)*. However, the present data suggest that the gene may be under some form of regulation because concentrations of mRNA decreased as the cycle advanced. Although the promoter regions of the human and mouse MARCKS gene contain numerous potential regulatory elements, none have been identified that regulate its transcription *(14)*. The reduction in luteal MARCKS mRNA and protein coincides

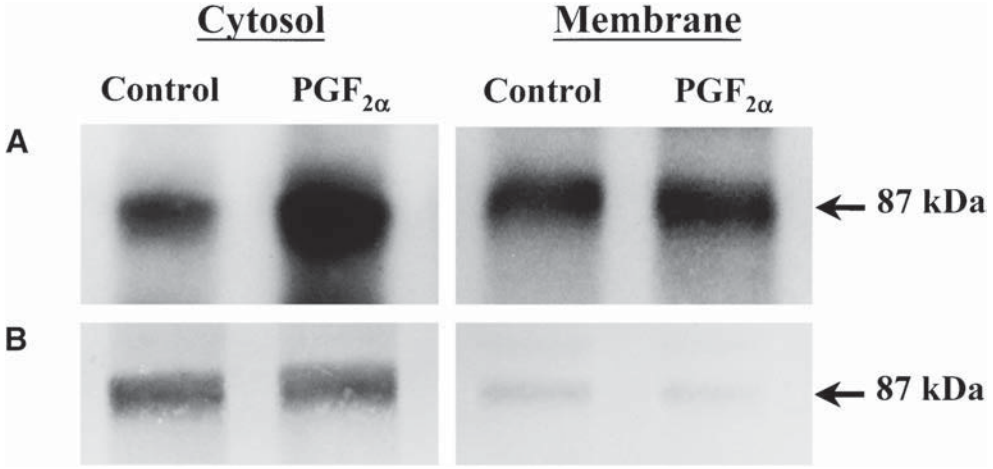

Fig. 3. Densitometric analysis of autoradiograph of SDS gel containing phosphorylated MARCKS protein in the cytosolic and membrane fractions of control and $PGF_{2\alpha}$-treated luteal slices on d 8 of the cycle **(A)** and the corresponding total MARCKS protein present in each of these fractions **(B)**.

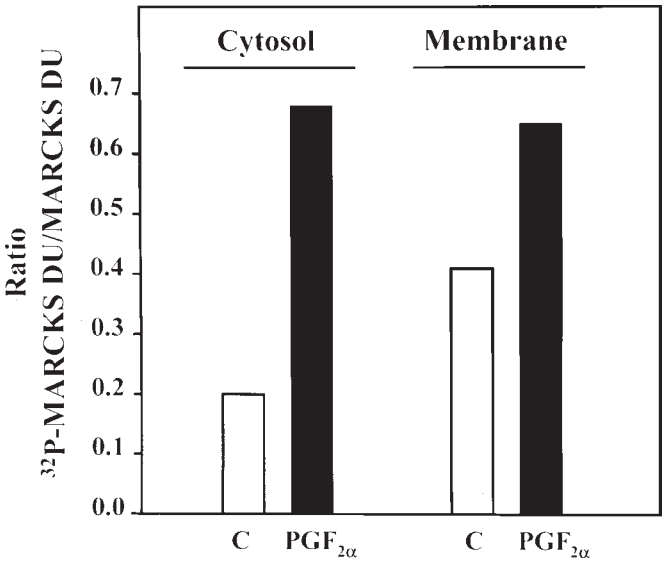

Fig. 4. Graphic representation of the ratio of arbitrary densitometric units of [^{32}P]-MARCKS protein to transferred protein for subcellular fractions of control (C) and $PGF_{2\alpha}$-treated luteal slices from a heifer on d 8 of the estrous cycle. DU, densitometric units.

with the increased uterine secretion of $PGF_{2\alpha}$ that causes luteal regression and thus terminates the cycle *(15,16)*. It is conceivable that the pulsatile secretion of $PGF_{2\alpha}$ that normally provokes luteal regression ultimately suppresses MARCKS gene expression or promotes mRNA instability. This is supported by the data of Brooks et al. *(17)*, who found that MARCKS protein and mRNA levels were significantly decreased several hours after treatment of Swiss 3T3 cells or mouse fibroblasts with phorbol ester, which, like $PGF_{2\alpha}$, is known to activate PKC. The same type of reduction in MARCKS mRNA and protein has been observed in response to treatment of cells with physiological activators of PKC such as bombesin and platelet-derived growth factor *(18)*.

On the other hand, the observed reduction in MARCKS mRNA and protein may be owing to expression of this gene in only the large luteal cell, which produces oxytocin as well as progesterone. This seems like a logical possibility if the MARCKS protein is involved in facilitating the exocytosis of oxytocin because the percentage of large luteal cells of the total steroidogenic cell population is maximal by d 8–12 of the cycle and decreases thereafter *(19,20)*. Although cell-specific expression of the MARCKS gene within a particular endocrine gland (organ) composed of heterogeneous secretory cell types has not been hitherto documented, it has been found that organ-specific expression of this gene does occur *(14)*.

$PGF_{2\alpha}$ has been shown to activate bovine luteal PKC *(5,21)*, the only kinase that specifically phosphorylates MARCKS protein *(22)*. Furthermore, in vitro exposure of MC3T3-E1 osteoblasts to $PGF_{2\alpha}$ or phorbol 12-myristate 13-acetate stimulated activation of PKC and phosphorylation of MARCKS *(23)*. Phosphorylation adds at least three molecules of phosphorus to the highly basic phosphorylation domain of MARCKS, reducing its electrostatic charges *(24)*. As a consequence of this reduced charge, membrane-bound phosphorylated MARCKS loses its ability to crosslink actin filaments, is translocated to the cytoplasm, and hence contributes to disassembly of the cytoskeletal actin cortex and promotes exocytosis of secretory granules *(11)*. In the present study, the evidence indicates that in vitro exposure of bovine luteal tissue to $PGF_{2\alpha}$ promotes phosphorylation of MARCKS protein, which is detectable in the cytosolic fraction of tissue homogenates. Because of the association of phosphorylated MARCKS with the plasma membrane, it is conceivable that the protein was translocated to the cytoplasm. Such a translocation of phosphorylated MARCKS during the 10-min exposure to $PGF_{2\alpha}$ is consistent with the time frame during which this eicosanoid has been shown to stimulate the luteal secretion of oxytocin *(5)*.

Materials and Methods

Experiment 1

Twelve beef heifers were utilized in an experiment to measure MARCKS mRNA in corpora lutea during d 4, 8, 12, and 16 of the estrous cycle. On each of the specified days of the cycle, corpora lutea were removed from each of three heifers by colpotomy *(5)* and were quartered and immediately immersed in liquid nitrogen. The corpora lutea were subsequently stored at –80°C until assayed for mRNA. For this experiment, as well as those described subsequently, all procedures involving animals were performed in accordance with the institutional guidelines for the care and use of animals.

RNA Extraction and Northern Blotting

Luteal tissue samples (0.5 g) were pulverized in liquid nitrogen with a cold mortar and pestle. Total RNA was isolated using TRIzol Reagent (Gibco-BRL, Grand Island, NY) and a monophasic solution of phenol and guanidine isothiocyanate *(25)*, and was recovered in 50 μL of diethylpyrocarbonate (DEPC)-treated water. Quantity and quality were estimated spectrophotometrically. For Northern blot analysis, 30 μg of total RNA from each sample was denatured by heating at 60°C for 15 min in sample preparation buffer (1 part 10X 3[*N*-morpholino]propanesulfonic acid [MOPS]) buffer [0.2 *M* MOPS, 80 m*M* Na acetate, pH 7.0], 1.75 parts deionized formaldehyde, and 5 parts deionized formamide) at a ratio of 9:31, and then chilled on ice. Denatured RNA was mixed with 2 μL of gel-loading buffer (50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol FF in DEPC-treated water) and loaded onto 1.1% agarose gels containing 6% formaldehyde and 0.5 μg/mL of ethidium bromide. Duplicate gels were run for increased accuracy, and total RNA from bovine brain was extracted and included as a positive control. Gels were subjected to electrophoresis in 1X MOPS running buffer at 30 V overnight, and then washed for 1 h with DEPC-treated water. Gels were then soaked in 6X saline sodium citrate (SSC) for 15 min (1X SSC = 0.15 *M* NaCl, 15 m*M* sodium citrate, pH 7.0), and RNA was transferred onto Nytran Plus nylon membranes (Schleicher & Schuell, Keene, NH) using capillary transfer overnight. Membranes were soaked in 5X SSC for 10 min, and RNA was UV crosslinked (1200 μJ) to the membranes. The membranes were incubated for 6 h at 42°C with 200 μL/cm^2 of prehybridization solution: 50% deionized formamide; 5X SSC; 50 m*M* K_2PO_4, pH 8.0; 5X Denhardt's solution (0.1% each bovine serum albumin [BSA], Ficoll, and polyvinylpyrrolidone [PVP]; Sigma, St. Louis, MO), 100 μg/mL of denatured salmon testes DNA for hybridization (Sigma), and 0.1% SDS.

Probes for MARCKS and 18S rRNA were prepared as follows. The template for MARCKS was a 1.698-kb fragment excised from a Bluescript plasmid construct containing MARCKS cDNA (a gift from Dr. P. J.Blackshear, National Institute of Environmental Health Sciences [NIEHS]) using a *Pst*I digest. The probe for 18S rRNA was constructed using a *Bam*HI restriction digest of pT7 RNA 18S (Ambion, Austin, TX) and a *Hind*III linearized plasmid containing 80 bp of the human 18S rRNA gene. Probes were made from the DNA templates by random hexanucleotide priming with [^{32}P]dCTP (Dupont New England Nuclear, Boston, MA) as the radioactive label (Prime-a-Gene Labeling System, Promega, Madison, WI). Unincorporated label was removed by passage through a Sephadex G-50 column (Quick Spin columns, Boehringer-Mannheim, Indianapolis, IN).

Hybridization solution (50 μL/cm^2 membrane) (50% deionized formamide; 5X SSC; 20 m*M* K_2PO_4, pH 6.5; 1X Denhardt's solution [0.1% each BSA, Ficoll, and PVP; Sigma], 100 μg/mL of denatured salmon testes DNA for hybridization [Sigma], and 0.1% SDS) was mixed with the labeled probe to obtain approx 1.5–3 × 10^6 cpm/mL. After overnight incubation at 42°C in an automated rotation hybridization oven (Robbins Scientific, Sunnyvale, CA), membranes were washed with 2X SSC and 0.1% SDS at room temperature for 30 min, and then washed with 2X SSC and 0.1% SDS at 50°C for 30 min. Some membranes were washed further with 0.1X SSC and 0.1% SDS at 50°C for 30 min. Blots were exposed to a storage phosphoscreen (Molecular Dynamics, Sunnyvale, CA) overnight or for several days. Screens were scanned by a PhosphorImager SI and visualized with ImageQuaNT software (Molecular Dynamics). Between probing for MARCKS and 18S rRNA, membranes were stripped with 50% formamide, 6X SSPE (1X SSPE = 0.18 *M* NaCl; 10 m*M* sodium phosphate; and 1 m*M* EDTA, pH 7.4) for 30–45 min at 65°C.

Signal densities were quantified with ImageQuaNT using volume quantitation of equal areas for any given signal. Background correction was computed by the local average method. Values for MARCKS mRNA as a percentage of 18S rRNA for each sample loaded were calculated. These values were then normalized to d 4 MARCKS mRNA and plotted for the remaining days of the cycle.

Experiment 2

Twelve beef heifers were utilized in an experiment to measure changes in MARCKS protein during the estrous cycle. Corpora lutea were removed via colpotomy *(5)* from each of four heifers on d 8, 12, and 16 of the cycle. Because of difficulty in enucleating luteal tissue from the ovarian stroma during colpotomy on d 4 of the cycle, quantitation of MARCKS protein in corpora lutea at this stage of the cycle was not studied. After removal of the connective tissue capsule the corpora luteum was cut in half, placed into liquid nitrogen, and subsequently stored at –80°C until processed. Luteal tissue removed from one heifer on d 8 of the cycle was also utilized for a preliminary in vitro study described in "Response of Luteal MARCKS to $PGF_{2\alpha}$."

Aliquots of the frozen corpora lutea (250 mg) were homogenized in 7.5 mL of buffer A (50 m*M* Tris-HCl, pH 8.3; 5 m*M* EDTA, 0.15 *M* NaCl, 5 μ*M* [4-{2-aminoethyl}benzene sulfonylfluoride, HCl]), and the homogenate was centrifuged at 1000*g* for 10 min at 4°C. The resulting supernatant was placed into a boiling water bath for 10 min to precipitate heat-unstable proteins (MARCKS protein is heat stable; *[26]*), after which it was centrifuged at 15,000*g* for 10 min (4°C). The protein concentration of the supernatant was determined using the Pierce BCA protein assay (Pierce, Rockford, IL). Aliquots of 200 μg of total protein from each sample were placed in a microcentrifuge tube to which was added 200 μL of immobilized protein G agarose beads. The combined contents were allowed to incubate for 1 h at 25ºC with gentle mixing. Samples were centrifuged at 1000*g* and washed five times with 400 μL of precipitation buffer (20 m*M* sodium phosphate, pH 7.5; 500 m*M* NaCl; 0.1% SDS; 0.5% deoxycholic acid, 0.02% sodium azide). The precipitated protein pellet was resuspended in 60 μL of SDS loading buffer (0.125 *M* Tris-HCl, pH 6.8; 4% SDS, 0.1% bromophenol blue; 20% glycerol; 10% β-mercaptoethanol) plus 1 μg of ovalbumin and boiled for 5 min. These samples were centrifuged in a microcentrifuge at 14,000*g* for 2 min at 25°C, and the supernatants were collected and subjected to 7.5% SDS polyacrylamide gel electrophoresis-(PAGE). Molecular weight markers were electrophoresed with the samples. Gels were silver stained and the appropriate bands subjected to densitometric scanning. Data are expressed as the densitometric MARCKS to ovalbumin ratio.

To confirm that the band on the gel equivalent to a molecular mass of 87 kDa was MARCKS protein, luteal protein extracts from heifers were also subjected to Western blotting. Luteal proteins (300 μg total per corpus luteum) were subjected to electrophoresis as already described and transferred to Nytran Plus nylon membranes for immunoblotting according to the methods of Towbin et al. *(27)*. An equivalent quantity of bovine brain protein was utilized for comparative purposes. To reduce nonspecific binding, membranes were incubated for 2 h (25°C) with 5% nonfat dry milk dissolved in water. Following this, blots were incubated with MARCKS 2F12 monoclonal antibody (1:500 in 2.5% nonfat dry milk) (Upstate Biotechnology, Lake Placid, NY) for 1 h at 25ºC, after which a 1:2000 dilution of alkaline phosphatase (AP) conjugated goat antimouse IgG secondary antibody (Bio-Rad, Hercules, CA) was added and incubation continued for another hour. MARCKS bands were visualized using an AP development kit (Bio-Rad).

Response of Luteal MARCKS to $PGF_{2\alpha}$

In a preliminary study, luteal tissue collected from one heifer on d 8 of the cycle was maintained at 4°C and sliced (0.3 mm thickness), and aliquots of the slices (221 ± 7 mg) were placed into two flasks containing 2 mL of phosphate-deficient Dulbecco's modified Eagle's medium (DMEM). The luteal slices were incubated for 2 h at 37°C in order to deplete the tissue of phosphate. After phosphate starvation, [^{32}P]-orthophosphate (200 μCi/mL) (Dupont New England Nuclear, Boston, MA) was added to each treatment flask and incubation continued for 1 h. Flasks containing slices were then treated with ethanol vehicle (control: 15 μL) and $PGF_{2\alpha}$ analog (56 n*M* Cloprostenol) (Bayer, Shawnee Mission, KS) and incubation continued for 10 min. After stopping the reaction by placing the flasks on ice, tissue slices were washed twice with 3 mL of DMEM. Slices were then homogenized with a Tekmar Tissuemizer (Tekmar, Cincinnati, OH) in 1 mL of buffer A containing enzyme inhibitors (5 μ*M* microcystin, 1 μ*M* calpeptin, 1X protease inhibitor cocktail set I) (Calbiochem, La Jolla, CA). The homogenate was centrifuged at 1000*g* for 10 min (4°C) to remove nuclei. To acquire cytosolic and membrane fractions, the supernatant was centrifuged at 100,000*g* for 1 h (4°C). The resulting supernatant represented the cytosolic fraction. The pellet (membrane fraction) was resuspended in 0.5 mL of buffer A containing 1% Nonidet-P40 and stored overnight (4°C). This membrane suspension was centrifuged at 100,000*g* for 1 h (4°C) to acquire the supernatant containing the membrane proteins. The subcellular fractions were placed into a boiling water bath as described above to obtain heat-stable MARCKS protein. The protein content of the sample was determined by the Pierce BCA protein assay and equal amounts of sample from the cytosolic (150 μg/lane) or membrane fraction (100 μg/lane) were subjected to 7.5% SDS-PAGE.

Subsequently, the proteins were transferred to a nylon membrane over a 5-h period, and then subjected to autoradiography to expose the phosphorylated proteins. After obtaining an adequate autoradiographic image, membranes were immunoblotted with mouse monoclonal MARCKS 2F12 antibody (1:500) to locate the specific position of phosphorylated MARCKS. The protein-antibody complexes were probed with an antimouse IgG-AP secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). MARCKS bands were visualized after processing the membranes by using an alkaline phosphatase conjugate substrate kit (Bio-Rad). Densitometry readings were performed on MARCKS bands from the autoradiograph and corresponding bands from the Western blot using a densitometer (Molecular Dynamics). Data are expressed as the ratio of densitometric units of [^{32}P]-MARCKS to that of the transferred protein.

Statistical Analyses

Data on concentrations of MARCKS protein and mRNA were analyzed by use of one-way analysis of variance. When warranted by a significant *F* value, differences among means were tested for significance using the least significant difference test.

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