Spatiotemporal Interactions of Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) Protein with the Actin Cytoskeleton and Exocytosis of Oxytocin upon Prostaglandin $F_2a$ Stimulation of Bovine Luteal Cells

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ABSTRACT

In the bovine corpus luteum (CL) phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) protein in response to prostaglandin $F_2a$ ($PGF_{2a}$) is correlated with the secretion of oxytocin. The present study was conducted to 1) examine the intracellular translocation characteristics of wild-type and mutant forms of a green fluorescent protein (GFP)-conjugated MARCKS (MARCKS-GFP) after $PGF_{2a}$ treatment and 2) evaluate $PGF_{2a}$-induced temporal changes in MARCKS-GFP and actin cortex associated with exocytosis of oxytocin. In experiment 1, cells of the bovine CL were cultured on coverslips overnight. Then, wild-type and mutant MARCKS-GFP constructs were transfected separately into cells and expression was detected through fluorescence microscopy. Forty-eight hours after transfection, cells were treated with vehicle, $PGF_{2a}$ (56 nM), or a phorbol ester (12-O-tetradecanoylphorbol-13-acetate [TPA], 1 $\mu$M). Treatment of cells expressing wild-type MARCKS-GFP with $PGF_{2a}$ and TPA resulted in translocation of MARCKS from the plasma membrane to the cytoplasm within 2.5 min. Phosphorylation mutant MARCKS-GFP (m3) protein was localized on the plasma membrane, and treatments did not cause its translocation to the cytoplasm. Myristoylation mutant MARCKS-GFP (G2A) was observed solely in the cytoplasm, and no changes were detected in the intracellular location of this mutant MARCKS after treatment. In experiment 2, luteal cells were transfected with one of the three MARCKS-GFP constructs. Cells were then fixed and probed sequentially for oxytocin and fluorescent protein expression. Results revealed that only wild-type MARCKS-GFP transfected large luteal cells contained advanced signs of exocytosis (peripheral movement of oxytocin vesicles; shorter actin filaments) with translocation of MARCKS-GFP from plasma membrane to the cytoplasm in response to $PGF_{2a}$ treatment. These data demonstrate that phosphorylation of membrane-bound MARCKS protein is requisite for exocytosis of oxytocin to occur in bovine large luteal cells.

corpus luteum, mechanisms of hormone action, oxytocin, signal transduction

INTRODUCTION

Oxytocin is synthesized in hypothalamic nuclei, but in some mammalian species such as the cow this neuropeptide is also synthesized and secreted by large steroidogenic cells of the corpus luteum (CL) [1]. Secretion of this neuropeptide occurs by exocytosis, which involves transport of vesicles through a cytoskeletal matrix, including an actin cortex that is in close apposition with the plasma membrane [2]. The integrity of the cortex is maintained by the cross-linking of actin filaments by a number of proteins, including myristoylated alanine-rich C kinase substrate (MARCKS) protein [3, 4]. In addition to its cross-linking function, MARCKS also anchors the actin network to the inner leaflet of the plasma membrane through the myristoylated N-terminal domain [5]. Phosphorylation of MARCKS by protein kinase C (PKC) disrupts the cross-linking and anchoring capacity and causes translocation of MARCKS from membrane to cytoplasm [6]. Phosphorylation-dependent translocation of MARCKS has been found to be involved with the disassembly of the actin cortex in a variety of secretory cells [7, 8]. In the bovine CL, biochemical analyses have revealed that activation of PKC by prostaglandin $F_2a$ ($PGF_{2a}$) results in phosphorylation and translocation of MARCKS, events closely correlated with exocytosis of luteal oxytocin [9].

Although biochemical approaches have provided evidence for an association of actin disassembly with exocytosis of oxytocin in the bovine CL, there is a need to examine further the subcellular changes in actin cytoskeletal filaments and vesicle transport that characterize the exocytic process in this endocrine gland. This can be accomplished through stimulation of large luteal cells containing transfected green fluorescent protein (GFP)-conjugated MARCKS (MARCKS-GFP) constructs [10] and concomitant immunocytochemical tracking of oxytocin granules within large luteal cells. The present study was conducted to 1) examine cytologically the translocation of MARCKS-GFP in luteal cells in response to $PGF_{2a}$ stimulation and 2) evaluate the dynamics of oxytocin exocytosis that are concomitant with changes in the actin cytoskeleton as a consequence of MARCKS translocation.

MATERIALS AND METHODS

Luteal Cell Culture

Four beef heifers were checked twice daily for behavioral estrus (Day 0), using a vasectomized bull. The CL was collected on Day 8 of the cycle per vaginum under lidocaine (2%)-induced caudal epidural anesthesia [11]. All animal experimental procedures were reviewed and performed in accordance with the institutional Animal Care and Use Committee guidelines at Oregon State University. Upon removal, the CL was transported to the laboratory in cold (4°C) Ham F-12 medium (Life Technologies, Rockville, MD) supplemented with 44 mM NaHCO3, 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. The CL was cut in half, weighed, and minced into pieces approximately 2 mm3. Cell dispersion was achieved by incubating the minced tissue in 20 ml of F-12 medium.
with collagenase (3000 U/g of tissue; Worthington Biochemical Corp., Lakewood, NJ) for 2 h at 37°C. Dispersed cells were rinsed three times with F-12 medium. After centrifugation (1000 × g) cells were resuspended in 1 ml and at room temperature. After three washes with PBS, coverslips carrying cells were removed to air dry and then were mounted on glass slides using Prolong Antifade (Molecular Probes). Cells were examined using a fluorescent microscope, and images were recorded as described in the previous section. From each of four heifers, a total of 20 large luteal cells per treatment (vehicle or PGF2α) containing either transfected wt, m3, or G2A MARCKS-GFP were examined microscopically.

Statistical Analysis

Data on percentages of large luteal cells transfected with wt MARCKS-GFP exhibiting translocation of the protein in response to PGF2α, and TPA were analyzed by one-way ANOVA. Differences among means were tested for significance with the least significant difference test.

RESULTS

Translocation of MARCKS-GFP in Large Luteal Cells

The translocation of wt and mutant MARCKS-GFP was visualized by monitoring the changes in GFP fluorescence. Apparently, all three types of the plasmids were intensely expressed by the large luteal cells, as determined by the abundant GFP signal (Fig. 1). Treatment of cells expressing transfected wt and mutant MARCKS-GFP with ethanol (vehicle control) produced no changes in the distribution of the GFP-attached MARCKS protein (Fig. 1, A, D, and G; wt, m3, and G2A, respectively). Treatment of cells expressing wt MARCKS-GFP with PGF2α and TPA resulted in translocation of MARCKS from the plasma membrane to the cytoplasm within 2.5 min (Fig. 1, B and C, respectively). The majority of the wt MARCKS-GFP protein translocated from the periphery of the cells and accumulated in the paracrine region by 5 min. The mean percentage of large luteal cells in which translocation of wt MARCKS-GFP occurred in response to stimulation with PGF2α or TPA was greater than that of controls (P < 0.001; Fig. 2). Phosphorylation mutant (m3) MARCKS-GFP expression was detected solely on the plasma membrane, and neither PGF2α nor TPA stimulation induced translocation of this mutated MARCKS protein from the membrane to the cytoplasm at either time period studied (Fig. 1, E and F). In transfected cells containing the myristoylation site-mutated MARCKS-GFP, the protein product remained exclusively in the cytoplasmic compartment, and treatments with PGF2α and TPA failed to effect a change in its subcellular localization after either 2.5 or 5 min (Fig. 1, H and I).

Correlation of Phosphorylation and Translocation of MARCKS with the Changes in Cross-Linked Actin Filaments and Oxytocin Localization

To understand the functional role of phosphorylation and translocation of MARCKS protein, along with MARCKS-GFP expression, cells were fixed and stained sequentially for oxytocin and filamentous actin with fluorescent probes of different emission-excitation wavelengths. Images from three different channels were combined to superimpose the three subcellular constituents of the observed cell; actin cytoskeleton (blue), oxytocin granules (orange-red), and MARCKS-GFP (green). Treatment of control cells with vehicle (ethanol) had no significant effect on translocation of wt MARCKS-GFP, movement of vesicles containing oxytocin, or changes in actin filament composition (Fig. 3A). Wild-type MARCKS-GFP was associated with the cell...
FIG. 1. Translocation of wt MARCKS-GFP and its mutant products. Primary luteal cells were transfected with one of the MARCKS-GFP constructs by lipofection. Cells expressing wt (A–C), m3 (D–F), and G2A (G–I) MARCKS-GFP were treated with vehicle (ethanol, 10 μl), PGF2α (56 nM), or TPA (1 μM). Effects of treatments were recorded by brief exposures to a camera at 0, 2.5, and 5 min after treatments. Bar = 5 μm.
regions [18]. One of these regions is an eight-residue domain characteristic of exocytosis of this nanopeptide.

Phosphorylation or myristoylation of MARCKS protein precedes the bovine large luteal cell. Mutations that prevent phosphorylation of the plasma membrane-associated functional addition of the 14-carbon myristate moiety. The third domain is a 25-amino acid basic effector domain that contains PKC phosphorylation sites. This region, known as the phosphorylation site domain, contains 12 or 13 positively charged Lys/Arg residues [6] and interacts electrostatically with the membrane. Myristoylation and phosphorylation site domains provide hydrophobic and electrostatic interactions of MARCKS with the plasma membrane, respectively [6]. Phosphorylation of MARCKS by PKC disrupts its cross-linking and anchoring capacities and causes its translocation from membrane to cytoplasm [6]. Phosphorylation-dependent translocation of MARCKS is a pivotal event associated with the disassembly of actin cortex in a variety of secretory cells [7, 8, 19].

Translocation of MARCKS has been visualized using GFP-tagged MARCKS cDNA constructs in living CHO-K1 cells in a study elegantly conducted by Ohmori et al. [10]. Wild-type MARCKS-GFP translocated rapidly from membrane to cytoplasm in response to TPA treatment. However, a MARCKS-GFP construct containing the mutated phosphorylation site domain remained associated with membrane and actin cortex after treatment with TPA [10].

In experiment 1 of the present study, bovine luteal cells were transfected successfully with wt or mutant MARCKS-GFP constructs to observe cytochemical changes in response to treatment with PGF2α. Cells expressing either type of MARCKS-GFP plasmid appeared solely green under the fluorescence microscope. Based on morphology, only large luteal cells were observed and recorded. The percentage of large luteal cells exhibiting translocation of MARCKS protein did not differ among those responding to PGF2α and TPA (P > 0.05; Fig. 2). However, the effects of PGF2α stimulation, as observed by the decrease in fluorescence intensity over time, appeared more profound than those of TPA stimulation (Fig. 1, B and C). We speculate that unlike TPA stimulation that activates PKC because of its structural resemblance to diacylglycerol (DAG), PGF2α in addition to activating PKC also increases intracellular free Ca2+ via formation of inositol 1,4,5-trisphosphate (IP3), which activates Ca2+-dependent F-actin-severing enzymes scinderin and gelsolin [20]. Treatment of chromaffin cells with phorbol esters causes only a partial disruption of the cortical actin cytoskeleton [21].

Experiment 2 was conducted to ascertain whether translocation of MARCKS protein was associated with obvious structural changes in the actin cortex and mobilization of oxytocin granules. Unlike experiment 1, where observations were made on living cells, for experiment 2 cells were fixed with 4% paraformaldehyde. Thus, cellular micrographs may appear slightly different in Figures 1 and 3. Within 5 min of PGF2α stimulation, release-ready oxytocin vesicles were scattered in the cytoplasm, usually in close proximity to the plasma membrane, and relatively fewer vesicles were detected in the paranuclear region (Fig. 2B). However, oxytocin immunoreactivity was particularly intense in the paranuclear region, and only limited movement of vesicles containing oxytocin was observed in PGF2α-treated cells expressing either type of mutant MARCKS-GFP construct. In these cells, the actin cytoskeleton appeared to be intact and consisted of long filamentous actin (Fig. 3, C and D). Because the actin filaments remained intact, one would expect to observe accumulation of oxytocin granules in PGF2α-stimulated cells just beneath the actin cortex. However, as visible in Figure 3C, the antici-

**FIG. 2.** Mean (±SEM) percentage of large luteal cells transfected with wt MARCKS-GFP in which stimulation with PGF2α or phorbol ester (TPA) resulted in translocation of the protein from the plasma membrane to the cytoplasm. Means with different letters differ significantly (P < 0.001).

Exogenous PGF2α has been shown to stimulate secretion of oxytocin from bovine luteal cells both in vivo [11, 13] and in vitro [14]. However, whether uterine PGF2α induces secretion of luteal oxytocin to promote luteolysis in the cow as apparently occurs in the ewe [15] is controversial. Although there is some evidence that episodic luteal secretion of oxytocin might contribute to luteal regression in the cow [16], more recent evidence suggests otherwise [17]. Nevertheless, the present study was conducted to determine, using MARCKS-GFP transfected primary luteal cell cultures, whether MARCKS protein plays any role in PGF2α-induced secretion of luteal oxytocin. Our data demonstrate that phosphorylation of the plasma membrane-associated MARCKS protein is essential for exocytosis of oxytocin by the bovine large luteal cell. Mutations that prevent phosphorylation or myristoylation of MARCKS protein preclude initial intracellular mobilization of oxytocin granules characteristic of exocytosis of this nanopeptide.

MARCKS protein contains three highly conserved regions [18]. One of these regions is an eight-residue domain in the amino terminal region of the protein that has unknown function (MARCKS homology 2 domain). Another region is the myristoylation site that directs the cotranslational addition of the 14-carbon myristate moiety. Another region is a 25-amino acid basic effector domain that contains PKC phosphorylation sites. This region, known as the phosphorylation site domain, contains 12 or 13 positively charged Lys/Arg residues [6] and interacts electrostatically with the membrane. Myristoylation and phosphorylation site domains provide hydrophobic and electrostatic interactions of MARCKS with the plasma membrane, respectively [6]. Phosphorylation of MARCKS by PKC disrupts its cross-linking and anchoring capacities and causes its translocation from membrane to cytoplasm [6]. Phosphorylation-dependent translocation of MARCKS is a pivotal event associated with the disassembly of actin cortex in a variety of secretory cells [7, 8, 19].
Multiple staining of primary luteal cells expressing MARCKS-GFP. For spatiotemporal comparisons, MARCKS-GFP (green), oxytocin (orange-red), and actin (blue) signals were superimposed in their respective groups. Luteal cells were transfected with one of the MARCKS-GFP plasmids. Twenty-four hours after observation of fluorescence, cells were treated with vehicle (ethanol, 10 μl) or PGF$_2α$ (56 nM) for 5 min. This figure depicts the changes of wt MARCKS-GFP protein (A and B), phosphorylation-mutated MARCKS-GFP (C), and myristoylation-mutated MARCKS-GFP (D), oxytocin granules, and actin in response to treatments. Actin filaments appeared to be fragmented (arrows) at the periphery 5 min after PGF$_2α$ stimulation (B) compared with other groups where filaments remained intact (A arrows, C, and D). Bar = 5 μm.

Hence, no downstream signal would be generated. Trifaro et al. [23] suggested that exocytosis of secretory granules coincides with disassembly of actin filaments. Although various proteins bind and cross-link actin filaments and alter cytoskeletal dynamics, MARCKS possesses demonstrable properties that are correlated with the exocytotic process [10, 24].

The results of the present research confirm the existence of MARCKS protein in bovine large luteal cells and demonstrate that phosphorylation of the protein is essential for initiation of the exocytotic process. It appears from these observations that phosphorylation of MARCKS protein is involved with the generation of a signal propagated via the interior cytoskeletal matrix to ensure mobilization of secretory vesicles.
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REFERENCES