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Activation of Adenosine Monophosphate-Activated Protein Kinase Is an Additional Mechanism That Participates in Mediating Inhibitory Actions of Prostaglandin F_{2Alpha} in Mature, but Not Developing, Bovine Corpora Lutea[1]

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ABSTRACT

Elevated cytosolic calcium and protein kinase C are well-established mediators of luteolytic actions of prostaglandin F_{2alpha} (PGF_{2alpha}). The objectives of this study were to determine 1) if calcium/calmodulin-dependent kinase kinase 2 (*CAMKK2*) participates in mediating PGF_{2alpha} actions in developing (Day [d]-4) and mature (d-10) bovine corpus luteum (CL), 2) distal targets of CAMKK2, 3) developmental expression of adenosine monophosphate-activated protein kinase (AMPK), and 4) effects of AMPK activation on progesterone (P4) production. Expression of AMPK increased as the CL matured. Activation of the prostaglandin receptor (FP) induced rapid phosphorylation of AMPK, which was blocked by a CAMKK2 inhibitor. Changes in basal P4 secretion in vitro were determined in response to AMPK activation via metformin (met) or 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) in d-4 and d-10 CL. Production of P4 in d-10 CL decreased with met or AICAR compared to control, similar to activation by PGF_{2alpha}. Therefore, potential distal targets of AMPK in d-10 CL were examined during induced functional regression via exogenous PGF_{2alpha}. Serum and luteal P4 decreased at 2 and 4 h after administration of PGF_{2alpha}. Protein expression of LDLR decreased at 2 and 4 h, while those of ACAT1 and STAR increased 4 h after PGF_{2alpha}. During induced regression, alterations of cholesterol transport proteins contributed to decreased luteal and serum P4. Therefore, developmental differences in signal transduction associated with FP, specifically CAMKK2 and AMPK, partially contribute to differences in the ability of PGF_{2alpha} to induce regression in mature, but not developing, bovine CL. Multiple cholesterol transport proteins, including LDLR, were altered by PGF_{2alpha} and could be potential AMPK targets.

AMPK, bovine, corpus luteum, luteal regression, luteolysis, mechanisms of hormone action, progesterone, prostaglandins, protein kinases

INTRODUCTION

The ability of bovine corpus luteum (CL) to respond to luteolytic actions of prostaglandin F_{2alpha} ($PGF_{2\alpha}$) increases with time after ovulation in cattle. Developing CL (Day [d] 1 to d 5) fail to regress if a single exogenous bolus of $PGF_{2\alpha}$ is given, yet administration of the same dosage of $PGF_{2\alpha}$ to mature CL (d 6–15) will induce regression, with the cow returning to estrus within 48–72 h [1, 2]. The cellular mechanisms responsible for this developmental difference are unclear, yet lack of receptors [3–5] or ability to elicit a physiological response to $PGF_{2\alpha}$ [6–8] do not appear to explain these observations. Therefore, differences in the signal transduction mechanisms associated with the $PGF_{2\alpha}$ receptor (FP) might explain some of the characteristics of elicited responses to $PGF_{2\alpha}$ in mature versus developing CL.

In luteal cells, $PGF_{2\alpha}$ has effects by binding to its cognate G_q protein-coupled receptor and activating phospholipase C [9], which ultimately lead to increases in both cytoplasmic Ca^{2+} and activation of protein kinase C (PRKC). Indeed, activation of PRKC is thought to mediate the effects of a PGF_2-induced rise in cytoplasmic Ca^{2+} in luteal cells [10]. Developmental-specific expression of genes encoding distinct PRKC isoforms and genes participating in Ca^{2+} homeostasis have been implicated in cellular mechanisms of acquisition of luteolytic capacity by bovine CL [5, 11]. Interestingly, increased gene expression of calcium/calmodulin-dependent kinase kinase 2 (*CAMKK2*), a downstream target of Ca^{2+}, is more relevant in the activated signal transduction pathway in mature than in developing CL [11]. This observation implies that PRKC might not be the sole intracellular mediator of luteolytic actions of $PGF_{2\alpha}$. Whether CAMKK2 affects steroidogenesis directly or acts upon another intermediary step that impinges on luteal steroidogenesis is not known.

One potential intermediary step is adenosine monophosphate-activated protein kinase (AMPK), which is activated via phosphorylation by CAMKK2 [12]. This is a highly conserved, eukaryotic, heterotrimeric kinase involved in energy balance and composed of one catalytic subunit alpha (with two isoforms: *PRKAA1* and *PRKAA2*) and two regulatory subunits beta (with two isoforms: *PRKAB1* and *PRKAB2*) and gamma (with three isoforms: *PRKAG1*, *PRKAG2*, and *PRKG3*) [12]. AMPK is pharmacologically activated in response to the antidiabetic drug metformin [13] and by 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR), a cell-permeable, allosteric AMPK activator [14]. Activation of AMPK decreased progesterone (P4) secretion in murine, bovine, and primary F1 galline granulosa cells [15–17]. These results indicate a potential role of AMPK in P4 production in the bovine ovary. All subunits of AMPK and their isoforms were present in bovine CL [17], but the developmental expression

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and functional significance of AMPK in this tissue are unknown.

If CAMKK2 targets AMPK in bovine CL, increased gene expression of *CAMKK2* and *AMPK* in mature CL may lead to increased AMPK activation and, therefore, become an additional mechanism to mediate effects of $PGF_{2\alpha}$ at this developmental stage. Activation of AMPK may regulate P4 production by reducing high- and low-density lipoprotein receptors such as scavenger receptor class B member 1 (SCARB1) and low-density lipoprotein receptor (LDLR), which would decrease cholesterol uptake by steroidogenic cells. During luteal regression in primates, gene expression for lipoprotein receptors SCARB1 and LDLR decreased [18, 19]. The possibility that $PGF_{2\alpha}$ exerts luteolytic actions through AMPK regulation of cholesterol transport has yet to be explored in the bovine CL.

The objectives of the present study therefore were to explore alternative downstream components activated by the rise in cytosolic [Ca^{2+}] stimulated by $PGF_{2\alpha}$, such as AMPK, in developing and mature bovine CL. Furthermore, the potential identity of distal targets for AMPK, such as cholesterol transport mechanisms, when FP is activated during functional regression was explored. The specific aims were to determine 1) expression of AMPK in steroidogenic cells isolated from developing and mature CL, 2) phosphorylation of AMPK by activating FP on steroidogenic cells isolated from mature CL, 3) if a CAMKK2-specific inhibitor could abolish $PGF_{2\alpha}$-stimulated phosphorylation of AMPK, 4) if pharmacological agents that activate AMPK could mimic the effect of $PGF_{2\alpha}$ on P4 secretion in developing and mature CL, and 5) aspects of functional regression, indicated by marked decrease in P4 production, that could be suggested as potential targets for activated AMPK. Specifically, changes in protein expression in steroidogenic acute regulatory (STAR) and cholesterol transport proteins (LDLR, SCARB1, and ACAT1) were assessed. The overall hypothesis was that CAMKK2 and AMPK are components of the pathway subservient to the rise in concentration of cytoplasmic Ca^{2+} induced by $PGF_{2\alpha}$ in mature bovine CL, and that this developmental difference in signal transduction associated with FP can explain, at least in part, differences in the ability of $PGF_{2\alpha}$ to induce functional luteal regression in mature, but not developing, bovine CL.

MATERIALS AND METHODS

Animal Handling and Surgical Procedures

Nonlactating beef cows were observed twice daily for estrus at approximately 12-h intervals for 30 min per observation. The day when standing estrus was observed was designated as d 0. The West Virginia University Animal Care and Use Committee approved the protocol for the tissue collection (ACUC 01-0809). Ovaries or CL were collected via supravaginal incision under epidural anesthesia [20] with administration of 6–9 ml of 2% lidocaine (Butler Company).

Luteal Cell Dispersion and Slice Preparation

In the laboratory, developing (d-4) and mature (d-10) CL (n = 4 each) were dissected free of connective tissue, weighed, placed into cell dispersion medium (CDM; M-199 containing 0.1% bovine serum albumin [BSA], 25 mM Hepes, and 100 U/ml of fungicide), and cut into small, approximately 1 mm^3 fragments for experiment 1. The tissue fragments were processed for enzymatic tissue dissociation, and luteal endothelial cells were separated as previously described [7]. Briefly, magnetic tosyl-activated beads (Dynal Biotech) were used to separate endothelial cells and the nonadherent cells, and steroidogenic-enriched luteal cells were collected. The cell population designated as steroidogenic cells represented a heterogeneous population of cells, including fibroblasts, pericytes, lymphoid cells, and possibly a few endothelial cells not removed by the separation procedure. Cell viability and density were determined using Trypan blue exclusion and a hemocytometer; luteal cell viability was usually greater than 96%. In experiment 1, isolated steroidogenic cells were used only for mRNA expression in d-4 CL, whereas cells from d-10 CL were used for mRNA expression, phosphorylation of AMPK by activating FP and Western blots, and P4 measurements.

For experiment 2, d-4 and d-10 CL (n = 5 each) were again dissected free of connective tissue, weighed, and then cut into thin slices (weight, 1-2 mg; thickness, 0.5 mm) using a Thomas Stadie-Riggs Tissue Slicer (Thomas Scientific).

Semiquantitative Western Blot Analysis

Sample proteins were analyzed using semiquantitative Western blotting as previously described [21]. Briefly, protein samples from experiment 1 (n = 4; 10^5 isolated luteal steroidogenic cells) and experiment 2 (n = 5 d-10 CL from individual cows per hour) were obtained by homogenizing the luteal tissue in a buffer containing 50 mM Tris HCl, 150 mM NaCl, 1 mM ethyleneglycoltetra-acetic acid, 1 mM ethylenediaminetetra-acetic acid, 0.1% SDS, 1% Triton-X, protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 μg/μl of leupeptin, and 5 μg/μl of aprotinin), and phosphatase inhibitors (100 mM sodium fluoride, 2 mM sodium orthovanadate, and 10 mM sodium pyrophosphate). Sample protein concentration was determined using a Bio-Rad assay with BSA (Gibco BRL) as a standard. After concentrations were determined, 100 μg/lane of protein (STAR, PRKAA1, phosphorylated PRKAA [P-PRKAA], SCARB1, and LDLR) were loaded for experiment 3. Proteins were resolved with a 4%–15% gradient polyacrylamide TGX gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Millipore) using a Bio-Rad Mini Trans-Blot Cell.

For experiment 1, luteal cell samples were incubated at 37°C for 1, 2, 5, 10, or 20 min as described in detail below. Primary antibodies used included anti-rabbit AMPK (2532S; Cell Signaling Technology) and anti-rabbit P-PRKAA (2537S; Cell Signaling Technology), both used at 1:1000 (v/v), and mouse monoclonal to beta actin (AM4302; Ambion Life Technologies) at a dilution of 1:2000 (v/v). The secondary horseradish peroxidase-conjugated antibodies used were goat anti-rabbit antibody (for PRKAA and P-PRKAA) and goat anti-mouse antibody (beta actin) diluted to 1:10 000 (v/v) (both from Jackson ImmunoResearch). Images of the detected proteins were captured using the Fluorchem Q (ProteinSimple). Densitometry of the bands of interest was measured using Quantity One quantitation software. Relative P-PRKAA amount was calculated by normalization to AMPK and then subsequent normalization to actin in each sample.

For experiment 3, primary antibodies used included STAR antibody (bs-3670Rl; Bioss) at 1:250 (v/v), PRKAA antibody (2532S; Cell Signaling Technology) at 1:1000 (v/v), P-PRKAA antibody (2537S; Cell Signaling Technology) at 1:1000 (v/v), LDLR antibody (LS-C146979; LSBio) at 1:500 (v/v), SCARB1 antibody (ab24603; AbCam) at 1:1000 (v/v), and beta actin (A2228; Sigma-Aldrich) at 1:2000 (v/v). Samples were incubated overnight at 4°C with gentle shaking. Secondary antibodies included goat anti-mouse IRDye 680RD (for actin; 926-68170; Li-Cor) and goat anti-rabbit IRDye 800CW (for STAR, PRKAA, P-PRKAA, LDLR, and SCARB1; 827-08365; Li-Cor), both diluted to 1:10 000 (v/v). Band intensity was captured using Odyssey infrared imaging software and quantified through densitometry (ImageJ; National Institutes of Health). Signal intensity for each protein of interest was standardized to the corresponding intensity of beta-actin control in the same sample.

Experiment 1

Messenger RNA expression from d-4 (n = 4 individual CL) and d-10 (n = 4 individual CL) isolated steroidogenic cells was analyzed for the genes listed in Table 1 via real-time RT-PCR. Total RNA was isolated using TRI Reagent (Molecular Research Center) according to the manufacturer's instructions and as previously described [11]. Briefly, frozen CL tissue immersed in liquid nitrogen was pulverized mechanically using an RNase-free mortar and pestle. The pulverized tissue was homogenized in TRI Reagent using an RNase-free glass homogenizer. RNA samples were solubilized in RNase-free water. Total RNA concentration was determined via a spectrophotometer (ND-1000; NanoDrop Technologies). Threshold cycle (Ct) values were obtained and used in the mathematical model for relative quantification after normalization with the housekeeping gene, *GAPDH*.

Sample proteins were analyzed for P-PRKAA, PRKAA, and actin via Western blotting as described above. Specifically, dispersed luteal steroidogenic cells from d-10 CL (n = 4) were plated at 10^5 cells in 35 × 100-mm Petri dishes and treated with $PGF_{2\alpha}$ (1.0 μg/ml) alone or with $PGF_{2\alpha}$ and STO-609 (100 ng/ml; Tocris) and incubated at 37°C and 5% CO_2 in a humidified incubator for 1, 2, 5, 10, or 20 min. The reaction was terminated at each time point by aspirating the media, placing the plates on ice, and adding ice-cold protein lysis media. Media were again aspirated, and cells were removed with a

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TABLE 1. *Bos taurus* gene-specific primers and their sequences.

Gene	Primer[a]	GenBank Accession no.
PRKAA1	F: 5′-TGCACACATGAATGCAAAGA R: 5′-CATAGTTGGGTGAGCCACAA	NM_001109802
PRKAA2	F: 5′-CACGGTCCAGTTTGGATTCT R: 5′-TGAGACAGAGGACGACATGC	XM_583885
PRKAB1	F: 5′-CCACCACATCTCCTCCAAGT R: 5′-TGGTTCAACATGACGTGGTT	NM_001024558
PRKAB2	F: 5′-AAGGAGGCAAGGAGGTCTTC R: 5′-GTCCAGGATGGCAACAAAGT	XM_590219
PRKAG1	F: 5′-CCAGTTATTGACCCGGAATC R: 5′-TTGGGGAACTCGGTGATAA	NM_174586
PRKAG2	F: 5′-GCTGGAGAAATTCGAGTTGG R: 5′-TGGTTGGAACGATGTCGTAA	XM_580300
PRKAG3	F: 5′-TCACAGAGCAGCAGTTTCGT R: 5′-TGCTCGATGGTGAGAGCTAA	BC109945
GAPDH	F: 5′-AATATCATCCCTGCTTCTAGTGG R: 5′-CATACTTGGCAGGTTTCTCCA	NM_001034034.1

[a] F, forward; R, reverse.

rubber policeman cell scraper. Once removed, fresh lysis buffer was added and protein isolated as described above.

Dispersed luteal steroidogenic cells from d-10 CL (n = 4) were plated at 10^5 cells per well in 96-well, flat-bottom culture plates with 300 µl of Minimum Essential Medium (MEM; Nunc, Scientific Laboratory Supplies) at 37°C and 5% CO_2 in a humidified incubator. Cells were then treated with MEM, MEM plus PGF, media plus STO-609, or MEM plus $PGF_{2\alpha}$ plus STO-609, at the same concentrations listed above, overnight. Media were collected the following day and frozen until assayed for P4.

Experiment 2

Luteal slices, isolated as described above, from d-4 (n = 5) and d-10 (n = 5) CL were incubated in individual glass tubes (13 × 10 mm) with 500 µl of MEM (Life Technologies) and AMPK activators (described below) before hormones were added. Treatments included MEM (control), $PGF_{2\alpha}$ (1.0 µg/ml), metformin (10, 5, 1, 0.5, and 0.1 mM; New England Mail Order Pharmacy), or AICAR (7.5, 0.75, and 0.075 mM; Tocris). After 30 min, media were removed and fresh media added back along with treatments for 2 h of incubation at 37°C with shaking at 200 rpm. Progesterone was quantified by radioimmunoassay (RIA) as previously described [22]. After incubation, slices were homogenized using an Omni Tissue Homogenizer (Omni International) in incubation media and frozen at −20°C until assayed for P4.

Experiment 3

Cows (body weight, 450–700 kg) were assigned randomly among treatment groups: day 10, hour 0 (d10-h0, n = 5); day 10, hour 2 (d10-h2, n = 5), and day 10, hour 4 (d10-h4, n = 5). Cows received a subcutaneous injection of $PGF_{2\alpha}$ (25 mg; Lutalyse; Zoetis) or saline (d10-h0) at 0, 2, or 4 h before lutectomy. CL were weighed and divided into equal sections for protein isolation and measurement of luteal P4. Tissue for protein isolation was snap-frozen in liquid nitrogen and stored at −80°C; remaining tissue was transported to the laboratory in ice-cold saline. Tissue for luteal P4 was homogenized with an Omni Tissue Homogenizer and then frozen at −20°C until analysis of P4 via RIA. Blood samples were collected hourly via caudal venipuncture until CL excision. Samples were allowed to clot and then centrifuged at 3500 × *g* for 15 min, and serum was collected and stored at −20°C. Serum and CL were analyzed for P4 by RIA.

A two-color detection scheme was used to permit simultaneous probing for target proteins. Band intensity imaging was captured using Odyssey infrared imaging software and quantified through densitometry (ImageJ). Signal intensity for a protein of interest was standardized to the corresponding intensity of actin in the same sample. This normalization procedure for semiquantitative estimation of protein has been validated [21].

Statistical Analysis

Effects of $PGF_2\alpha$ and STO-609, AMPK phosphorylation, and luteal P4 content were examined using ANOVA with Dunnett post hoc test. Concentrations of P4 in serum and culture media were examined using ANOVA with repeated measures and Dunnett post hoc test. Two-way ANOVA followed by Tukey-Kramer test was used to determine statistically significant differences in mRNA encoding AMPK subunits between luteal developmental stages. Normalized protein concentrations ratios were tested for normal distribution via the Shapiro-Wilk test [23]. A one-tailed Student *t*-test was used for comparison of data sets that had a normal distribution. For data sets that did not have a normal distribution, a one-tailed Wilcoxon two-group test (Mann-Whitney test) was used. A *t*-test was used to compare the value of P4 at the highest dose of metformin and AICAR with control values at both d 4 and d 10. Data were analyzed using JMP Version Pro 11 and SAS Version 9.3 software (both from SAS Institute, Inc.). Significance criterion (alpha) for all tests was 0.05. Data are depicted as the mean ± SEM.

RESULTS

Experiment 1

Expression of all AMPK subunits was increased significantly in mature versus developing CL with the exception of AMPK gamma 1 (PRKAG1) and AMPK gamma 2 (PRKAG2) (Fig. 1). Activation of FP induced rapid phosphorylation of PRKAA, and the CAMKK2 inhibitor, STO-609, eliminated the phosphorylating effect of $PGF_{2\alpha}$ on PRKAA (Fig. 2). $PGF_{2\alpha}$ alone significantly reduced P4 accumulation in the mature CL. STO-609 had no effect alone but eliminated the reduction in P4 when incubated with $PGF_{2\alpha}$ ($P < 0.05$) (Fig. 3).

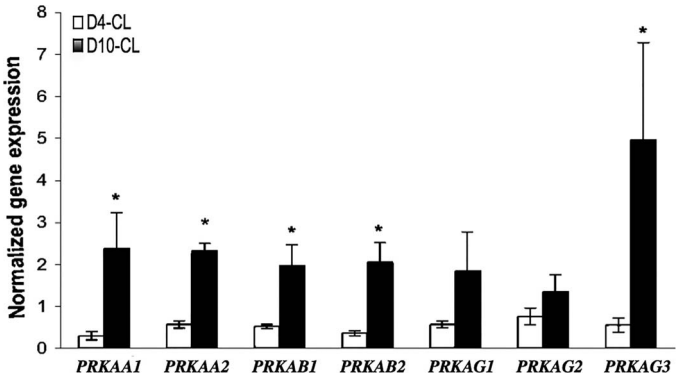

FIG. 1. Gene expression of AMPK in steroidogenic cells isolated from developing and mature bovine CL (n = 4 each). Asterisks indicate significant differences from d-4 CL ($P < 0.05$). With the exception of AMPK gamma 1 (PRKAG1) and AMPK gamma 2 (PRKAG2), mRNA expression of all other AMPK subunits was increased in the mature versus developing CL ($P < 0.05$). Threshold cycle (Ct) values were obtained and used in the mathematical model for relative quantification after normalization with the housekeeping gene, *GAPDH*. Data are shown as the normalized mean ± SEM.

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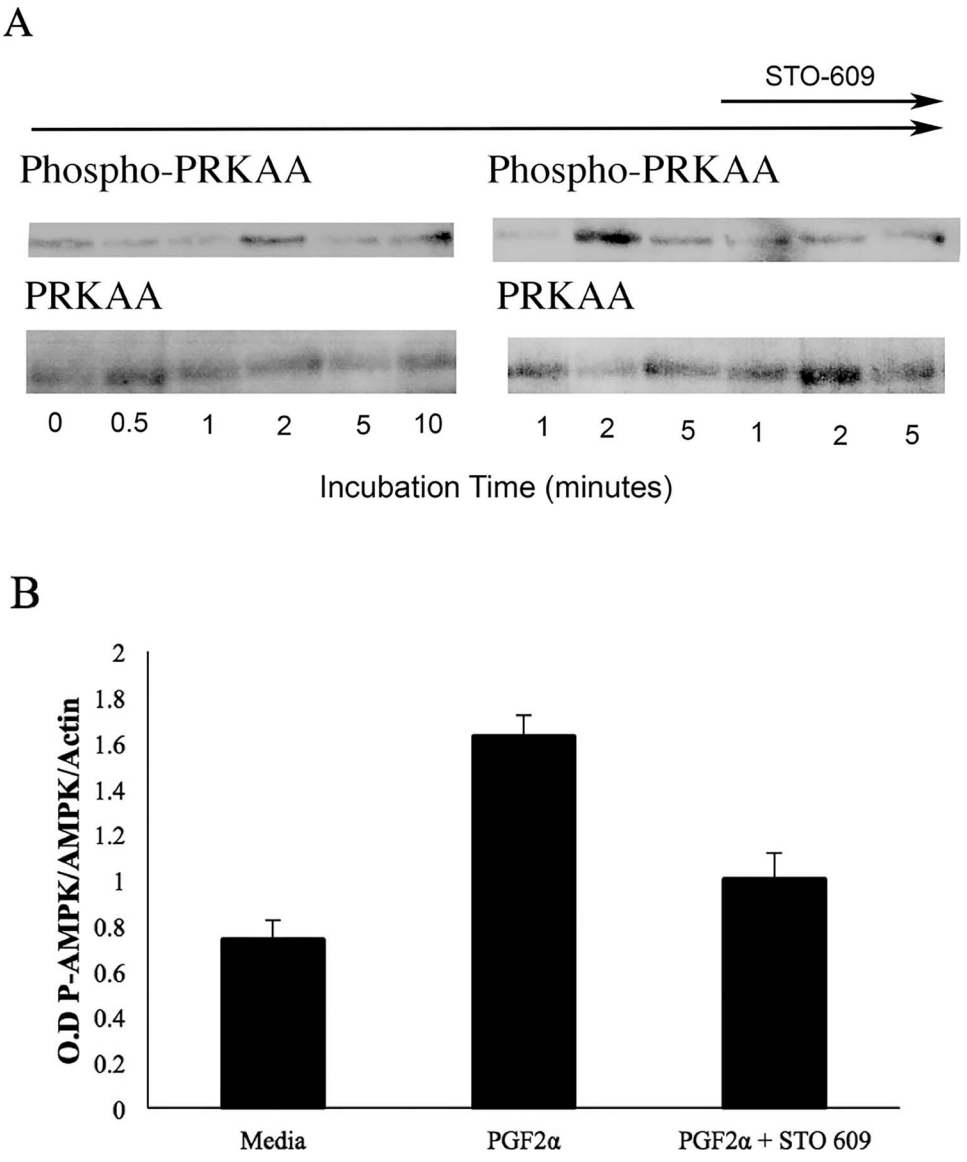

FIG. 2. Ability of $PGF_{2\alpha}$ to activate PRKAA in steroidogenic luteal cells isolated from mature bovine CL. **A**) Activation of FP induced rapid phosphorylation of PRKAA, and the CAMKK2 inhibitor, STO-609, abrogated this effect of $PGF_{2\alpha}$ on AMPK ($P < 0.05$, indicated by different letters in **B**). **B**) Cells were treated with media alone, $PGF_{2\alpha}$ (1.0 μg/ml) alone, or $PGF_{2\alpha}$ and STO-609 (100 ng/ml) for 1, 2, or 5 min. Samples of isolated proteins were analyzed via Western blotting by probing the blots with specific antibodies for P-PRKAA, PRKAA, and actin proteins. Relative quantification was calculated after normalization of optical density (o.d.) determined for P-PRKAA normalized to its corresponding AMPK and then subsequently to actin. The *y*-axis shows the mean ± SEM of the normalized o.d. determined for P-PRKAA divided by PRKAA and beta actin.

Experiment 2

No significant effect of metformin ($P = 0.91$) or AICAR ($P = 0.51$) at any concentration was observed in d-4 CL (Fig. 4A). A decrease in basal P4 was observed with both metformin (0.39 ± 0.20 ng/ml, 10 mM, $P = 0.006$) and AICAR (0.40 ± 0.15 ng/ml, 7.5 mM, $P = 0.0117$) in d-10 CL when compared to control (Fig. 4B).

Experiment 3

Decreases in serum P4 concentration at 2 h (2.08 vs. 2.96 ng/ml, $P = 0.054$) and 4 h (1.57 vs. 2.96 ng/ml, $P = 0.013$) were observed after administration of $PGF_{2\alpha}$. This change was also observed in luteal P4 content (d10-h0, 5.05 ng/mg; d10-h2, 2.03 ng/mg; d10-h4, 1.74 ng/mg; $P < 0.05$). Changes in protein expression of LDLR, SCARB1, PRKAA, STAR, and ACAT1 are shown in Figure 5. P-PRKAA was not detected. Decreases in LDLR protein expression were observed at 2 h (0.79 vs. 0.41 arbitrary normalized o.d., $P = 0.09$) and at 4 h (0.79 vs. 0.13 arbitrary normalized o.d., $P = 0.004$) after $PGF_{2\alpha}$ injection (Fig. 5A). Increases in ACAT1 occurred by 4 h (0.67 vs. 0.21 arbitrary normalized o.d., $P = 0.010$) after $PGF_{2\alpha}$ injection (Fig. 5B). A significant quadratic effect of time after $PGF_{2\alpha}$ was found for PRKAA protein expression ($P = 0.037$); however, expression at 2 and 4 h after $PGF_{2\alpha}$ administration did not differ from that at 0 h (Fig. 5C). An increase in STAR protein expression was observed 4 h (3.62 vs. 1.25, $P = 0.01$) after $PGF_{2\alpha}$ injection but not at 2 h (1.02 vs. 1.25) (Fig. 5D). No difference in SCARB1 protein expression occurred at either 2 h (1.92 vs. 1.74) or 4 h (2.75 vs. 1.74) after $PGF_{2\alpha}$ injection (Fig. 5E).

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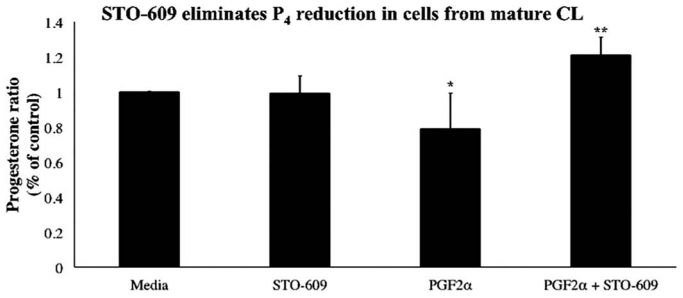

FIG. 3. The CAMKK2 inhibitor, STO-609, eliminated the effect of $PGF_{2\alpha}$ on P4 production in the mature, but not developing, bovine CL. Dispersed luteal cells were isolated and incubated in media alone, media plus $PGF_{2\alpha}$ (1.0 μg/ml), media plus STO-609 (100 ng/ml), or media plus $PGF_{2\alpha}$ plus STO-609. $PGF_{2\alpha}$ alone significantly reduced P4 accumulation in the mature CL. STO-609 had no effect alone but eliminated the reduction in P4 when incubated with $PGF_{2\alpha}$ ($P < 0.05$). *Significant effect of PGF2a in reducing basal progesterone accumulation ($P < 0.05$). **Significant effect of STO-690 in eliminating the inhibitory effect of $PGF_{2\alpha}$ on basal progesterone accumulation ($P < 0.05$).

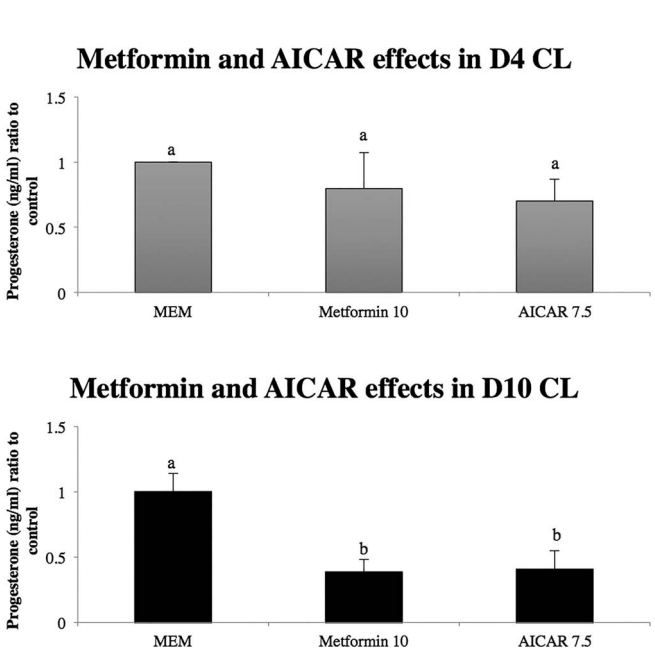

FIG. 4. At the greatest concentrations tested, metformin and AICAR each ($P < 0.05$) decreased basal P4 production in mature (d-10) bovine CL. Luteal slices were incubated for 30 min in MEM and metformin (10 mM) or AICAR (7.5 mM) without hormones, after which all media were removed and fresh MEM, metformin, or AICAR and hormones were added back. Slices were homogenized in the media and then collected and frozen until analyzed for P4 with RIA. Bars with different letters are significantly different ($P < 0.05$). Data obtained for d-4 and d-10 CL are shown in **A** and **B**, respectively.

DISCUSSION

Mature bovine CL had greater mRNA expression of AMPK subunits, rapid $PGF_{2\alpha}$-induced PRKAA phosphorylation that was ameliorated by a CAMKK2 inhibitor, and pharmacological AMPK activators that mimicked the inhibitory effect of $PGF_{2\alpha}$ on basal P4 secretion, none of which occurred in the developing bovine CL. This evidence indicates that both CAMKK2 and AMPK are involved in the signal transduction mechanism mediating inhibitory effects of $PGF_{2\alpha}$ on mature, but not developing, bovine CL. In addition, a CAMKK2 inhibitor, STO-609, blocked the inhibitory effect of $PGF_{2\alpha}$ on basal P4 secretion in dispersed, mature bovine luteal cells.

In rodents, activation of a Ca^{+2}/calmodulin-dependent effector system plays a role in mediating antisteroidogenic actions of $PGF_{2\alpha}$ [24]. Stocco et al. [24] showed that $PGF_{2\alpha}$-induced expression of the transcription factor *nur77* through Ca^{+2}/calmodulin-dependent activation of the ERK/MAPK pathway reduced P4 secretion in the rat. This precedent, in conjunction with evidence presented in the current study, supports the conclusion that in addition to PRKC activation [10], CAMKK2 and AMPK, two downstream targets of Ca^{+2}/calmodulin activation, are likely involved in P4 synthesis/secretion by $PGF_{2\alpha}$ in bovine CL. Additionally, the mature bovine CL is capable of responding to $PGF_{2\alpha}$ with a Ca^{+2} signal of greater magnitude than the developing CL [5], which may result in increased activation of CAMKK2 and, subsequently, AMPK. In the mature bovine CL, *CAMKK2* expression was almost doubled when cows were treated with $PGF_{2\alpha}$, with no differences in expression found in the developing CL [11]. These observations support the finding from the present study that CAMKK2 plays a integral role in the signal transduction mechanism of the mature, but not the developing, CL in response to FP activation.

Data on expression of *AMPK* mRNA support and expand upon studies in which *AMPK* expression was measured in whole bovine ovaries, small and large follicles, oocytes, and CL [15]. Expression of *PRKAA1, PRKAA2, PRKAB1, PRKAB2,* and *PRKAG3* was greater in mature than in developing CL. Similarly, the present results agree with those of Tosca et al. [15–17], who provided evidence in the rat, hen, and cow that AMPK is involved in steroid production in granulosa cells. It is not surprising that AMPK plays a role in steroidogenesis in luteal cells as a proportion of this population is derived from granulosa cells, which are known to acquire some luteal features when cultured [25]. Importantly, the present results indicate a difference in *AMPK* gene expression between developing and mature CL, with increased expression in mature bovine CL. Therefore, activation of AMPK should have a greater effect in the mature than in the developing CL. Activation of AMPK using metformin decreased P4 production in bovine granulosa [17] and luteal cells [26]. In experiment 2, no significant changes were observed in P4 production when d-4 luteal tissue was treated with metformin or AICAR. However, d-10 CL responded to both AMPK activators with a significant inhibition in P4 production. Interestingly, metformin, which is the most widely used pharmaceutical for treatment of type II diabetes, is prescribed to nearly 120 million people worldwide [13] and has also been used to treat polycystic ovary syndrome (PCOS) in women who were overweight or insulin resistant. Metformin directly affects the ovaries and also decreases insulin resistance in patients with PCOS. Data presented here support the idea that metformin is able to directly affect steroidogenesis in luteal tissue, which may have implications for the use of metformin during conception and gestation. Collectively, these data demonstrate that luteal function is regulated in the mature, but not the developing, CL, at least in part by the signal transduction pathway involving CAMKK2 and AMPK.

Present results corroborated that activation of FP by exogenous $PGF_{2\alpha}$ causes a decrease in serum and luteal P4 concentrations [3, 6, 27, 28]. Serum and luteal P4 were decreased as early as 2 h after $PGF_{2\alpha}$ and remained decreased at 4 h. However, no changes in serum P4 were observed at 1 h after $PGF_{2\alpha}$ (data not shown), which adds to the established time frame of functional regression after exogenous injection of $PGF_{2\alpha}$ [3, 6, 27, 28]. Elevated concentrations of PRKAA

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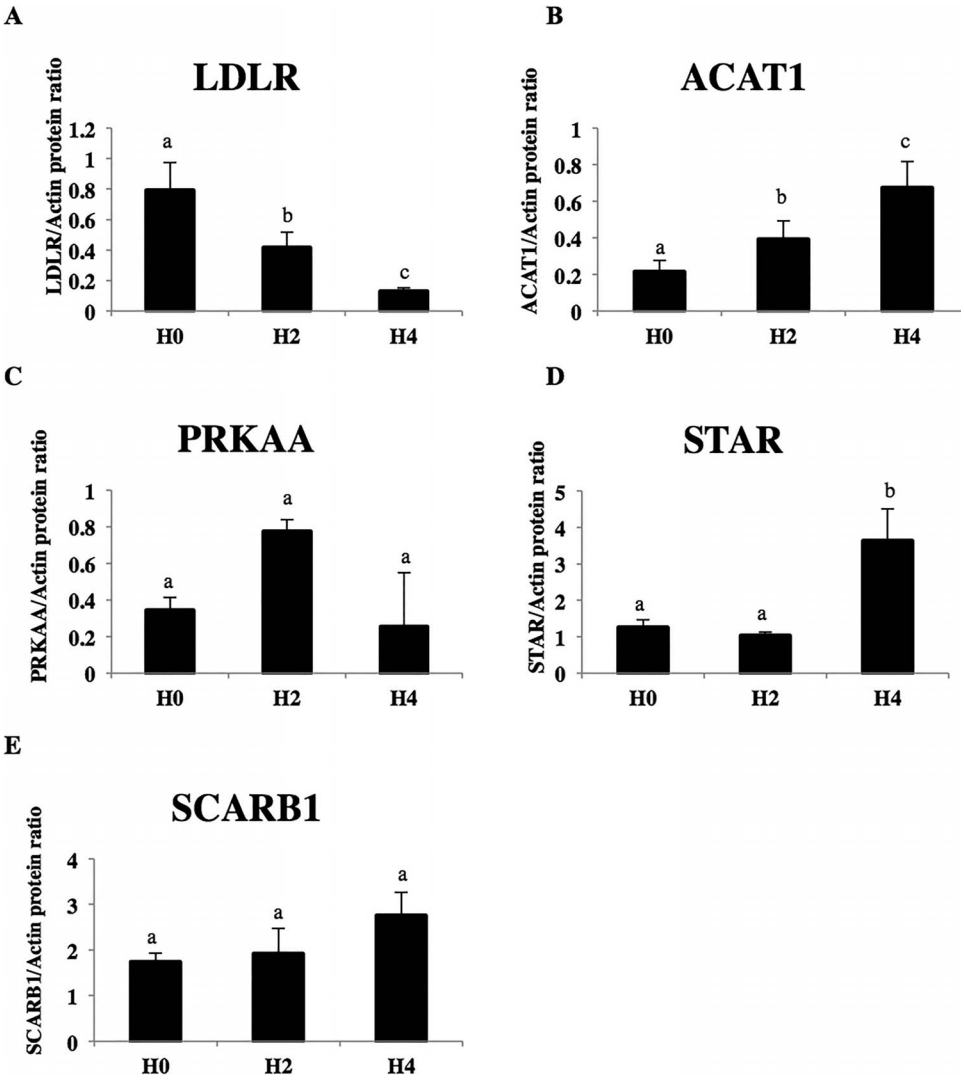

FIG. 5. Semiquantitative analysis of the densitometric data derived from Western blots for LDLR (**A**), ACAT1 (**B**), PRKAA (**C**), STAR (**D**), and SCARB1 (**E**). Protein samples were isolated from mature bovine CL at 0, 2, and 4 h after $PGF_{2\alpha}$. The *y*-axis shows the ratio of the optical density (o.d.) for the protein of interest corrected by the detected o.d. for its corresponding actin. Data are given as the mean ± SEM. Differing letters denote significant differences ($P < 0.001$) within each panel.

protein observed 2 h after FP activation may serve as a mechanism by which both serum and luteal P4 concentrations are decreased during functional regression. At 4 h after FP activation, P4 concentrations continued to decrease at the onset of structural regression. Whether AMPK continues to be required for structural regression is not clear. Phosphorylated PRKAA was not detectable at either 2 or 4 h after $PGF_{2\alpha}$. This may be a result of rapid PRKAA phosphorylation/dephosphorylation. Park et al. [29] reported PRKAA phosphorylation within 5 sec in skeletal muscle of rats, and in experiment 1, PRKAA phosphorylation occurred within 2 min and was rapidly dephosphorylated at 5, 10, and 20 min (data at 20 min not shown) in luteal tissue in vitro. Therefore, time points chosen after $PGF_{2\alpha}$ injection in experiment 3 may have been too late to observe any change in P-PRKAA.

During functional regression, cholesterol transport through LDLR was lower at 2 and 4 h after $PGF_{2\alpha}$, suggesting that substrate transport by these receptors was critical to the reduction of P4 production. Lower LDLR would lead to decreased cholesterol, the substrate for P4 production. Decreases in LDLR reduced 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), catalyzing a rate-limiting step in cholesterol production in vivo [30] and increasing ACAT1 in vitro [31]. Cells adjust to the number of LDL receptors in order to produce sufficient cholesterol for the metabolic needs of the cell without overaccumulation [32]. This function is key because cells are able to keep the concentration of unesterified cholesterol in membranes constant, whereas requirements and exogenous supply are fluctuating constantly [33]. Increases of ACAT1 indicate an attempt by the luteal tissue to compensate for decreases in cholesterol supply. No change in SCARB1 in mature CL was observed after $PGF_{2\alpha}$ administration. During spontaneous luteolysis in primate CL, changes in gene expression or protein concentrations of SCARB1 were not different between mid-late to functional late CL but were decreased from functional to functionally regressed late-stage CL [18, 19]. Functional late CL were defined as having been collected on d 14–16 of the luteal phase, and functionally regressed late CL were a subset of those CL from monkeys with serum P4 values less than 0.5 ng/ml [19]. Based on the criteria used by Bogan et al. [18, 19], the time points used in the current study after $PGF_{2\alpha}$ was given

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reflected functional regression, whereas the results reported by Bogan et al. reflected structural regression.

In summary, expression of *AMPK* subunits was upregulated in the mature bovine CL. Additionally, treatment of steroidogenic cells with $PGF_{2\alpha}$ induced rapid PRKAA phosphorylation, which was blocked by a CAMKK2 inhibitor, and the AMPK activators, metformin and AICAR, decreased basal P4 only in the mature CL. Activation of FP decreased luteal and serum P4 concentrations 2 h after exogenous administration of $PGF_{2\alpha}$. Alterations in cholesterol transport accounted, at least in part, for this reduction in P4 production. In conclusion, developmental differences in signal transduction associated with FP, specifically CAMKK2 and AMPK, contribute to differences in the ability of $PGF_{2\alpha}$ to induce functional luteal regression in mature, but not developing, bovine CL. Furthermore, cholesterol transport via LDLR might be the target of this luteal developmental difference.

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