BIOLOGY OF REPRODUCTION **76,** 368–378 (2007)
Published online before print 22 November 2006.
DOI 10.1095/biolreprod.106.055749

Effects of Metformin on Bovine Granulosa Cells Steroidogenesis: Possible Involvement of Adenosine 5′ Monophosphate-Activated Protein Kinase (AMPK)[1]

Lucie Tosca, Christine Chabrolle, Svetlana Uzbekova, and Joëlle Dupont[2]

Unité de Physiologie de la Reproduction et des Comportements, Institut National de la Recherche Agronomique, 37380 Nouzilly, France

ABSTRACT

In mammals, IGFs are important for the proliferation and steroidogenesis of ovarian cells. Metformin is an insulin sensitizer molecule used for the treatment of the infertility of women with polycystic ovary syndrome. It is, however, unclear whether metformin acts on ovarian cells. Adenosine 5′ monophosphate-activated protein kinase (AMPK) is involved in metformin action in various cell types. We investigated the effects of metformin on bovine granulosa cell steroidogenesis in response to IGF1 and FSH, and studied AMPK in bovine ovaries. In granulosa cells from small follicles, metformin (10 mM) reduced production of both progesterone and estradiol and decreased the abundance of HSD3B, CYP11A1, and STAR proteins in presence or absence of FSH (10^{-8} M) and IGF1 (10^{-8} M). In cows, the different subunits of AMPK are expressed in various ovarian cells including granulosa and theca cells, corpus luteum, and oocytes. In bovine granulosa cells from small follicles, metformin, like AICAR (1 mM) a pharmaceutical activator of AMPK, increased phosphorylation of both Thr172 of AMPK alpha and Ser 79 of ACACA (Acetyl-CoA Carboxylase). Both metformin and AICAR treatment reduced progesterone and estradiol secretion in presence or absence of FSH and IGF1. Metformin decreased phosphorylation levels of MAPK3/MAPK1 and MAPK14 in a dose- and time-dependent manner. The adenovirus-mediated production of dominant negative AMPK abolished the effects of metformin on secretion of progesterone and estradiol and on MAPK3/MAPK1 phosphorylation but not on MAPK14 phosphorylation. Thus, in bovine granulosa cells, metformin decreases steroidogenesis and MAPK3/MAPK1 phosphorylation through AMPK activation.

AMPK, cumulus cells, granulosa cells, metformin ovary, progesterone, signal transduction, steroid hormones

INTRODUCTION

In most mammals, growth factors, including insulin, IGF1 (insulin-like growth factor-1) and FSH (follicle-stimulating hormone) are hormones that regulate both mitogenesis and steroidogenesis of ovarian follicles cells [1]. The biguanide metformin has been reported to enhance the sensitivity of peripheral tissues to insulin or IGF1 [2]. Metformin treatment associated with FSH induction of ovulation modulates the ovarian response resulting in more monofollicular cycles in women [3]. Thus, metformin may limit multiple pregnancies associated with assisted reproductive techniques [3]. Metformin has been used clinically for over several years in women with polycystic ovary syndrome [PCOS, 4]. PCOS involves hyperandrogenaemia and anovulation, associated with reproductive and metabolic disorders including insulin resistance, which may affect folliculogenesis and ovulation. In women with PCOS, metformin increases ovulation, fertilization, and pregnancy rates [5, 6]. However, its mechanism of action is still unclear. Metformin treatment may increase not only insulin sensitivity in peripheral tissues [7, 8] but also act directly on ovarian cells by decreasing the production of androgens and steroids [9–11]. Metformin enhances the action of insulin on the in vitro developmental potential of porcine oocytes and embryos [12]. The effects of metformin and its molecular mechanism in bovine ovarian cells have never been investigated. As for women with PCOS, metformin might be used to treat dairy cows with cystic ovarian follicles. We therefore examined the effects of metformin in normal granulosa cells.

In rat granulosa cells, metformin reduces both basal and FSH-induced progesterone secretion through the activation of adenosine 5′ monophosphate-activated protein kinase (AMPK) [13]. AMPK is a key regulatory enzyme in cellular energy homeostasis [14]. It is a heterotrimeric enzyme, consisting of one catalytic subunit, α (PRKAA1 and PRKAA2; formerly known as AMPK isoforms α_1 and α_2), and two regulatory subunits, β (PRKAB1 and PRKAB2; formerly known as AMPK isoforms β_1 and β_2) and γ (PRKAG1, PRKAG2, and PRKAG3; formerly known as AMPK isoforms γ_1, γ_2, and γ_3) [15]. Phosphorylation of threonine 172 of the α_1 subunit by upstream kinases, including STK11 (LKB1) and Calmodulin kinase kinase, is essential for its activity [15, 16]. The enzyme is activated by a change in the AMP/ATP ratio caused by exercice [17], hypoxia [18], hormones [19, 20], metformin [13, 21], or pharmacological drugs including 5-aminoimidazole-4-carboxamide-riboside-5-phosphate (AICAR) [22]. AMPK phosphorylates target proteins involved in a number of metabolic pathways, including lipid and cholesterol metabolisms (adipocytes, liver and muscle), glucose transport, and glycogen and protein metabolisms (see review [23]). Recently, we characterized AMPK in rat ovary and demonstrated that its activation reduces progesterone secretion through the MAPK3/MAPK1 pathway in response to AICAR in rat granulosa cells [24]. In mouse oocytes, microinjection of constitutively active AMPK stimulates meiotic resumption in dbcAMP-arrested oocytes, implicating AMPK in oocyte maturation [25–28]. In bovine species, seven *AMPK* genes have been mapped to six different chromosomes [29] and the genes *PRKAG3* and *PRKAG1* have been well characterized [30, 31]. However, the presence and the role of AMPK in bovine ovaries are still unknown.

The aims of this study were to investigate the effects and the molecular mechanism of metformin on bovine granulosa cells steroidogenesis (production of both progesterone and estradi-

[1]Supported by the GIS-AGENAE Programme, ANR and APIS-GENE. L.T. is a doctoral student supported by the Région Centre.
[2]Correspondence: FAX: 33 2 47 42 77 43; e-mail: jdupont@tours.inra.fr

Received: 17 July 2006.
First decision: 17 August 2006.
Accepted: 8 November 2006.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Oligonucleotide primer sequences used in this study.

Primer	Sequence	Accession no.	Product size (bp)
PRKAA1			
Sense	5′- CCT TTG GCA GTT GCC TAC CA -3′	NM206907	500
Antisense	5′- GAT CCC GAT CTC TGT GGA GT -3′		
PRKAA2			
Sense	5′- ACC AGG TGA TCA GCA CTC CA -3′	XM583885	445
Antisense	5′- CCT CGG ATC TTC TTA AAG AG -3′		
PRKAB1			
Sense	5′- CCA ACG GTG TTT CGA TGG AC -3′	BC001823	460
Antisense	5′- TGG GCT CAG GAA GCA AAG CT -3′		
PRKAB2			
Sense	5′- CAT GGC AGC AGG ATT TGG AG -3′	XM590219	360
Antisense	5′- GGT CTC GAC AAG ATG TCT CT -3′		
PRKAG1			
Sense	5′- GGT GTA CCT GCA GGA CTC CT -3′	DQ133597	600
Antisense	5′- AGG ATA TCC GAG AGG GAG AC -3′		
PRKAG2			
Sense	5′- GCT TCG TAG GAA TGC TCA CA -3′	BC079017	620
Antisense	5′- GGT CTC CAG TGT TTC CAG CT -3′		
PRKAG3			
Sense	5′- CAC AAC AGA ACG AGA CAG CC -3′	BC109945	300
Antisense	5′- TGT AGC ATG GTG TCG AAG AT -3′		
ACTIN			
Sense	5′- ACG GAA CCA CAG TTT ATC ATC -3′	D12816	188
Antisense	5′- GTC CCA GTC TTC AAC TAT ACC -3′		

ol). We also examined the involvement of AMPK in the effects observed.

MATERIALS AND METHODS

Hormones and Reagents

Purified ovine FSH-20 (oFSH) (lot no.AFP-7028D, 4453 IU/mg, FSH activity = 175 times activity of oFSH-S1) used for culture treatment was a gift from the National Hormone Pituitary Program (NIDDK, NIH, Bethesda, MD). Recombinant human IGF1 and metformin were from Sigma Chemical Co. (St. Louis, MO). Metformin was dissolved in sterile water. Taq DNA polymerase was provided by Promega (Madison, WI). McCoy A modified culture medium, penicillin, and streptomycin were purchased from Invitrogen (Cergy Pontoise, France). Thymidine methyl-H^3 was purchased from Perkin Elmer Life and Technological Sciences (Boston, MA).

Antibodies

Rabbit polyclonal antibodies to phospho-PRKAA Thr172, ACACA, phospho-MAPK3/MAP1 (Thr202/Tyr204), phospho-MAPK14 (Thr180/Tyr182), phospho-AKT (Ser473), and AKT were purchased from New England Biolabs Inc (Beverly, MA). Rabbit polyclonal antibodies to MAPK1 (C14) and to MAPK14 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to PRKAA1/2, PRKAA1, and phospho-ACACA Ser79 were obtained from Upstate Biotechnology Inc (Lake Placid, NY). Rabbit polyclonal antibodies to CYP11A1, STAR, and HSD3B were generously provided by Dr. Dale Buchanan Hales (University of Illinois, Chicago, IL) and Dr. Van Luu-The (CHUL Research Center and Laval University, Canada), respectively. Mouse monoclonal antibody to VINCULIN (VCL) was obtained from Sigma (St. Louis, MO). All antibodies were used at 1/1000 dilution in western blotting.

Animals and Isolation and Culture of Bovine Granulosa Cells

All procedures were approved by the Agricultural Agency and the Scientific Research Agency and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Adult cow ovaries were collected at the slaughterhouse and removed aseptically and transferred to culture medium. Granulosa cells from small follicles (<6 mm) were harvested by puncturing the follicles allowing expulsion of the cells. Cells were recovered by centrifugation, washed with fresh medium, and counted in a haemocytometer. The culture medium used was McCoy 5A supplemented with 20 mmol/L Hepes, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine (3 mmol/l), 0.1% BSA, 0.1 μmol/l androstenedione, 50 μg/l insulin, 5 mg/l transferrin, 20 μg/l selenium, and 10% FBS. The cells were initially cultured for 48 hours with no other treatment and then incubated in fresh culture medium with or without test reagents for the appropriate time. All cultures were performed under a water-saturated atmosphere of 95% air/5% CO_2 at 37°C.

Thymidine Incorporation into Granulosa Cells

Granulosa cells (2×10^5 viable cells/500 μl) were cultured in 24-well dishes in McCoy 5A medium and 10% FBS for 48h and were then serum starved for 24h. Next, 1 μCi/μl of [^{3}H]-thymidine (Amersham Life Science, Arlington Heights, IL) was added in the presence or absence of metformin (10 mM) and/or FSH (10^{-8} M) and IGF1 (10^{-8} M). Cultures were maintained at 37°C under 5% CO_2 in air. After 24 hours of culture, excess of thymidine was removed by washing twice with phosphate-buffered saline (PBS), and the cells fixed with cold trichloroacetic acid 50% for 15 min and lysed by addition of 0.5 N NaOH. The radioactivity was determined in scintillation fluid (Packard Bioscience) by counting in a β-photomultiplier.

Adenoviruses and Infection of Bovine Granulosa Cells

Dominant negative AMPK adenovirus (Ad-DN) was constructed from PRKAA1 carrying the Asp-157 to Ala (D157A) mutation as previously described [32]. Recombinant adenovirus was propagated in HEK293 cells, purified by caesium chloride density centrifugation, and stored as previously described [32]. Bovine granulosa cells were pre-infected with 100 pfu/cell adenovirus in serum-starved McCoy 5A for 16 h and then cultured for 36 h with 100 pfu/cell adenovirus and in the presence or absence of FSH (10^{-8} M), IGF1 (10^{-8} M) and/or metformin (10 mM). Preliminary studies revealed that within 16 h of infection (100 pfu/cell) with a green fluorescent protein (GFP)-expressing virus, 70 percent of granulosa cells expressed green fluorescent protein.

RNA Isolation and Real-Time PCR

Total RNA was extracted from ovaries (Ov), dissected small (<0.6 mm, SF) and large follicles (>0.8 mm, LF), corpus luteum (CL), and oocytes (Oo) using Trizol reagent according to the manufacturer's instructions (Invitrogen, Cergy Pontoise, France). RNA was quantified by measuring the absorbance at 260 nm and then treated with DNases to eliminate genomic DNA contamination. Samples were stored at −80°C until use. Real-time PCR was used to assay *PRKAA1*, *PRKAA2*, *PRKAB1*, *PRKAB2*, *PRKAG1*, *PRKAG2*, and *PRKAG3* mRNAs. Total RNA (1 μg) from bovine ovary, small and large

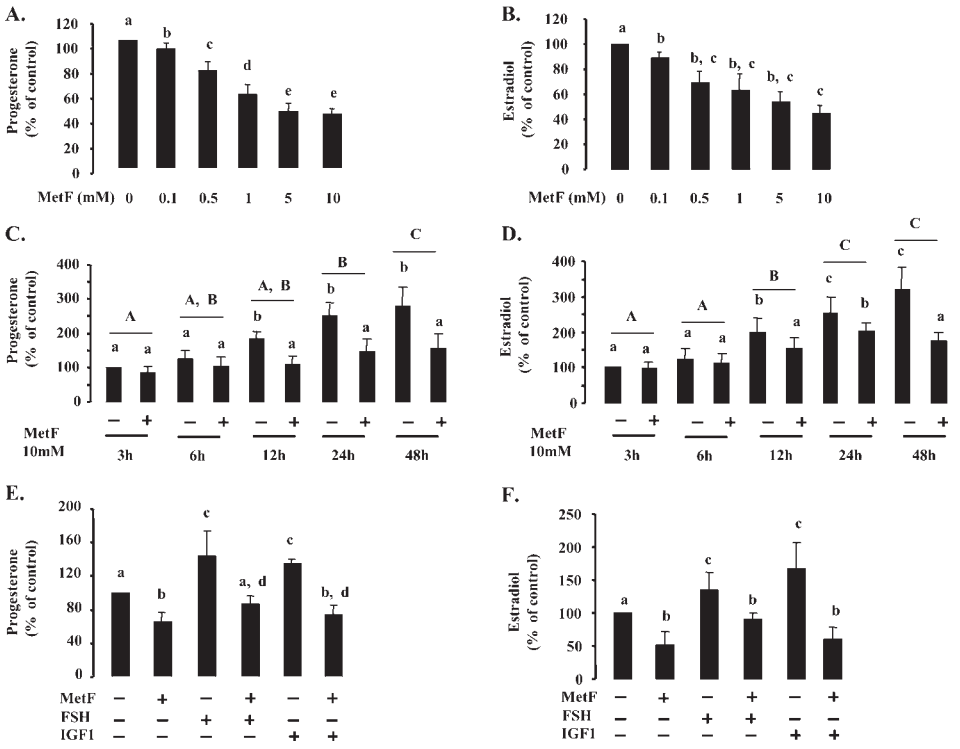

FIG. 1. Effect of metformin treatment on basal and FSH- or IGF1-stimulated secretion of progesterone and estradiol by bovine granulosa cells. Granulosa cells from small bovine follicles were cultured for 48h in medium with serum and then in serum-free medium in the presence or in the absence of various doses of metformin (**A** and **B**) for 48h, or for various times with 10 mM metformin (**C** and **D**), or in presence or absence of 10 mM metformin ± 10^{-8} M FSH or ± 10^{-8} M IGF1 (**E** and **F**) as described in *Materials and Methods*. The culture medium was then collected and analyzed for progesterone (**A**, **C**, and **E**) and estradiol (**B**, **D**, and **F**) content by RIA. Results are expressed as ng/ml/100µg and then as a percentage of the control value (i.e., basal conditions without metformin, FSH or IGF1). Results are means ± SEM of three independent experiments. Bars with different letters are significantly different ($P < 0.05$). For **C** and **B**, different capital letters indicate a significant effect of the time of stimulation whereas lower case letters indicate a significant effect of the metformin treatment.

follicle, corpus luteum, and oocyte samples was reverse transcribed in a 20 µl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 200 mM of each deoxynucleotide triphosphate (Amersham, Piscataway, NJ), 50 pmol oligo(dT)15, 5 IU ribonuclease inhibitor, and 15 IU Moloney murine leukaemia virus reverse transcriptase. RT reactions were carried out at 37 °C for 1 h. Single-strand cDNAs were amplified with specific sets of primer pairs designed to amplify parts of the different *AMPK* isoforms as described in Table 1. PCRs were carried out using 2 µl of the RT reaction mixture in a volume of 50 µl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer, and 1 UI Taq polymerase. The samples were processed for 35 PCR cycles (95°C for 1 min, 58°C for 1 min and 72°C for 1 min), with a final extension step at 72°C for 10 min. PCR products were visualized in agarose gel (1.5%) stained with ethidium bromide, and the DNAs were extracted from the agarose using the QIAEX II gel extraction kit (QIAGEN, Hilden, Germany) and sequenced in both directions using a Dye terminator kit and an ABI Prism

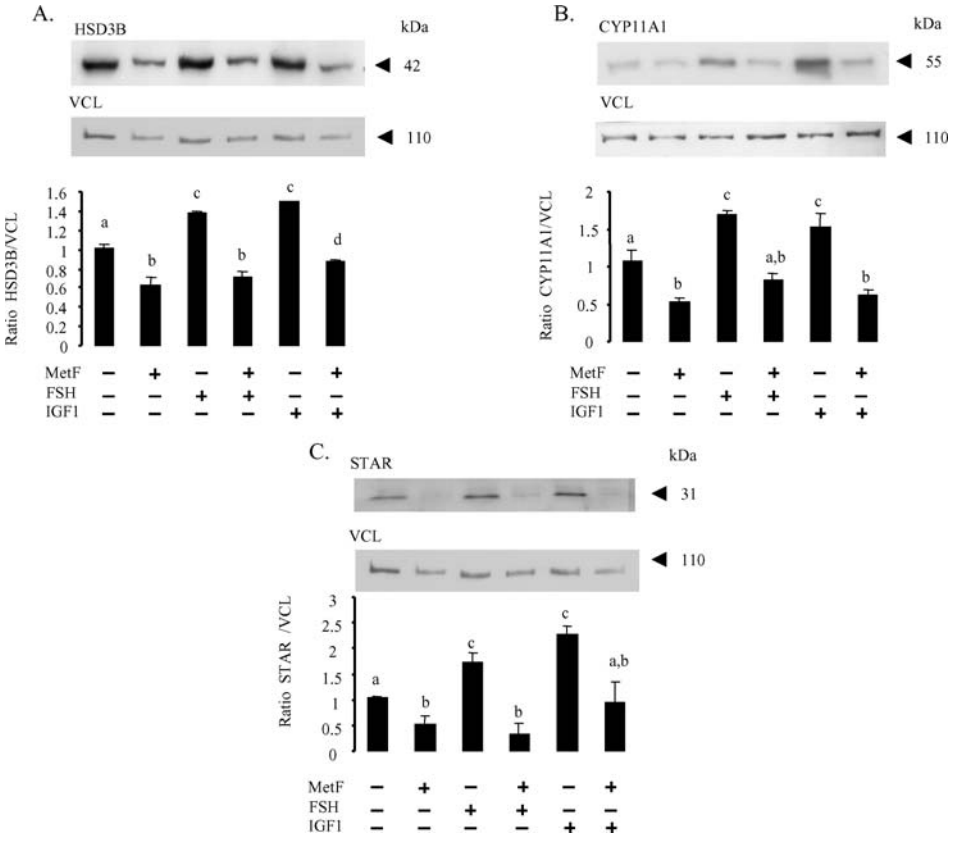

FIG. 2. Effect of metformin treatment on the amounts of the HSD3B, CYP11A1, and STAR proteins in bovine granulosa cells. Protein extracts from bovine granulosa cells cultured for 48h in the presence or in the absence of 10 mM metformin ± 10^{-8} M FSH or ± IGF1 were subjected to SDS-PAGE as described in *Materials and Methods*. The membranes were incubated with antibodies raised against the HSD3B (**A**), CYP11A1 (**B**), and STAR (**C**) proteins. Equal protein loading was verified by reprobing membrane with an anti-VCL antibody. Results are representative of at least three independent experiments. Blots were quantified and the HSD3B, CYP11A1 and STAR/αVCL ratios are shown. The results are expressed as means ± SEM of three independent experiments. Bars with different letters are significantly different ($P <$ 0.05).

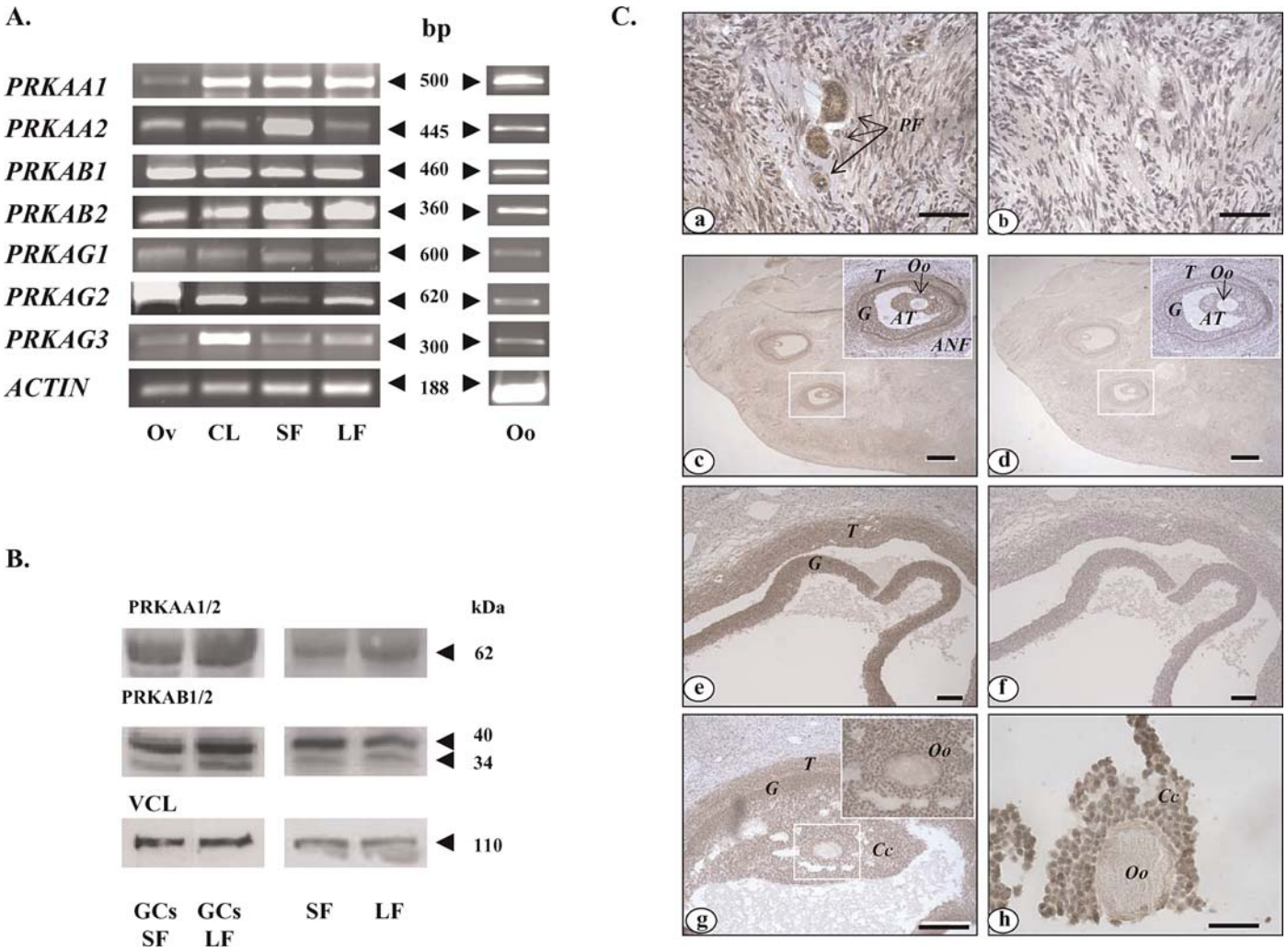

FIG. 3. AMPK characterization in bovine ovary. **A**) RT-PCR analysis of the mRNAs for *AMPK* regulatory (*PRKAB1/PRKAB2* and *PRKAG1/PRKAG2/PRKAG3*) and catalytic (*PRKAA1/PRKAA2*) subunits in whole bovine ovary (Ov), in small (SF) and large (LF) follicle, corpus luteum (CL) and oocyte (Oo). **B**) Detection of the PRKAA1 and 2 and PRKAB1 and B2 proteins by immunoblotting in SF and LG and in granulosa cells from SF and LG. Vinculin is used as a loading control (n = 3). **C**) Localization of PRKAA1 isoform in bovine ovary by immunohistochemistry. DAB-immunoperoxidase staining was performed on paraffin-embedded bovine ovary using antibodies against PRKAA1 (**a**, **c**, **e**, **g**, and **h**) or no primary antibodies (**b**, **d**, and **f**). Immuno-specific staining is brown. The sections were counterstained with haematoxylin. PRKAA1 is detected in primary follicles (**a**, PF) and antral follicles (**c**, ANF). Note also that PRKAA1 is observed in granulosa (G) and theca (T) cells and in cumulus cells (Cc) and oocyte (Oo) (**c**, **e**, **g**, and **h**). AT: antrum. White rectangles in **c**, **d**, and **g** encompass the regions which are magnified in inserts. Bars = 500 μm (**c** and **d**); 100 μm (**e** and **f**); and 50 μm (**a**, **b**, **g**, and **h**).

automated sequencer, model 377 (Biomolecular Research Facility, University of Virginia, Charlottesville, VA). PCR amplifications with only RNA were performed in parallel as negative controls (data not shown).

Western Blot

Total protein was extracted from cultured granulosa cells, whole ovary or dissected small and large follicles on ice in lysis buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Igepal) containing various protease inhibitors (2 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin) and phosphatase inhibitors [100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, (Sigma, l'Isle d'Abeau Chesnes, France)]. Lysates were centrifuged at 13 000 g for 20 min at 4°C, and the protein concentration in the supernatants was determined using a colorimetric assay (kit BC Assay, Uptima Interchim, Montluçon, France).

Cell extracts were subjected to electrophoresis on 10% (w:v) SDS-polyacrylamide gel under reducing conditions. Proteins were then electro-transferred onto nitrocellulose membranes (Schleicher and Schuell, Ecquevilly, France) by application of 80 volts for 1h30. Membranes were incubated for 1h at room temperature with Tris-buffered saline (TBS, 2 mM Tris-HCl, pH 8.0, 15 mM NaCl, pH 7.6), containing 5% nonfat dry milk powder (NFDMP) and 0.1% Tween-20 to saturate non-specific sites. Then, membranes were incubated overnight at 4°C with appropriate antibodies (final dilution 1:1000) in TBS containing 0.1% Tween-20 and 5% NFDMP. After washing in TBS-0.1% Tween-20, the membranes were incubated for 2 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (HRP, final dilution 1:10 Diagnostic Pasteur, Marnes-la-Coquette, France) in TBS-0.1% Tween-20. After washing in TBS-0.1% Tween-20, the signal was detected by ECL (enhanced chemiluminescence, Amersham Pharmacia Biotech, Orsay, France). The films were analyzed and signals quantified with Scion Image software (Fuji PhotoFilm).

Immunohistochemistry

Biopsies from ovaries or groups of COCs were fixed for 2h in solution containing 50% of saturated picric acid, 3.7% formaldehyde, and 5% of acetic acid. After serial dehydration steps, samples were embedded in paraffin and 7 μm-thick serial sections were cut. Sections were deparaffined, rehydrated, microwaved for 5 min in antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France), and then left to cool to room temperature. Sections were washed in a PBS bath for 5 min, and immersed in peroxidase blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (Dako Cytomation; Dako, Ely, UK). After three washes in a PBS bath for 5 min, sections were blocked with 5% goat serum in PBS for 20 min, and incubated overnight at 4°C with PBS/0.1% BSA containing anti-PRKAA1 antibody. After three washes for 5 min in a PBS bath, sections were incubated

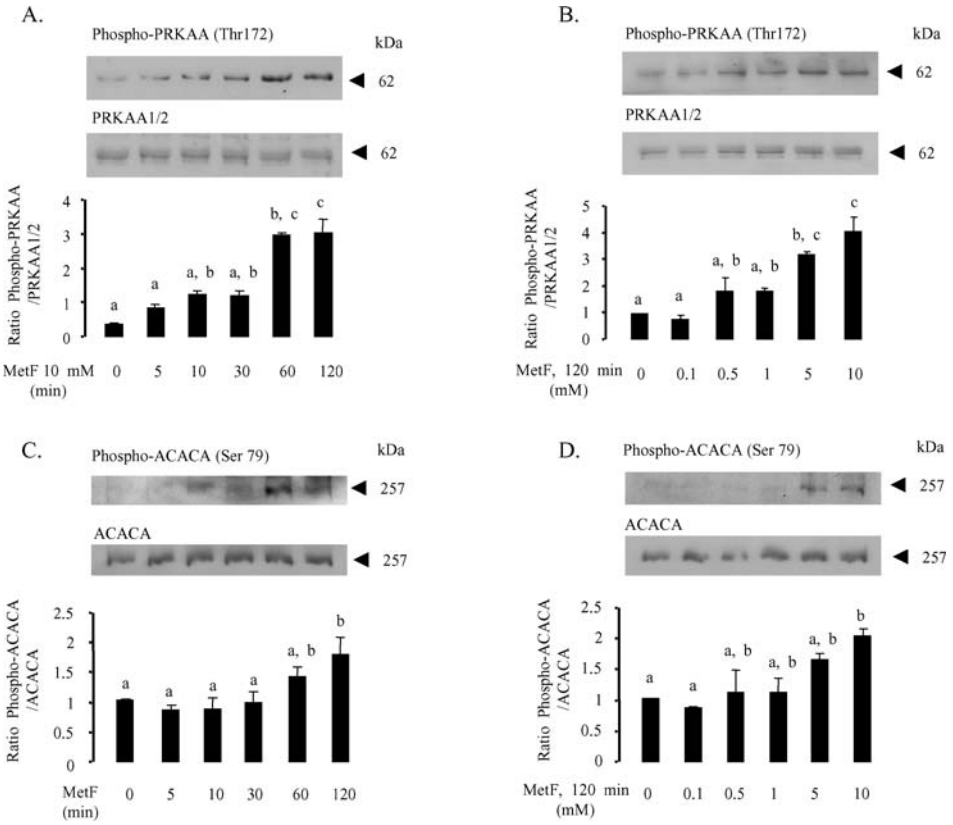

FIG. 4. Effect of metformin treatment on the phosphorylation of PRKAA and ACACA in bovine granulosa cells. Granulosa cell lysates were prepared from cells incubated with 10 mM metformin for various times: 0, 5, 10, 30, 60, or 120 min. Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-PRKAA and anti- PRKAA1/2 (**A**) or anti-phospho-ACACA and anti-ACACA (**B**) antibodies. Representative blots from three independent experiments are shown. Bands on the blots were quantified and the phosphorylated protein / total protein ratio is shown. The results are reported as means ± SEM. Bars with different letters are significantly different ($P < 0.05$).

for 30 min at room temperature with a biotinylated goat anti-mouse antibody. Then, after serial washing in PBS and incubation in streptavidin peroxidase solution (Lab Vision Corporation, Fremont, CA) for 10 min, staining was revealed by incubation at room temperature with 3,3′-diaminobenzidine (Lab Vision Corporation, Fremont, CA). The slides were counterstained with Meyer's haematoxylin (Sigma, St Louis, MO) and mounted in Depex (Sigma, St Louis, MO). Negative controls included replacing primary antibodies with rabbit IgG. Slides were observed using an Axioplan Zeiss transmission microscope.

The specificity of the antibody in rat tissues was confirmed by preincubating primary antibody with its blocking peptide (20 μg/ml SC-19128P from Santa Cruz for PRKAA1, [24]).

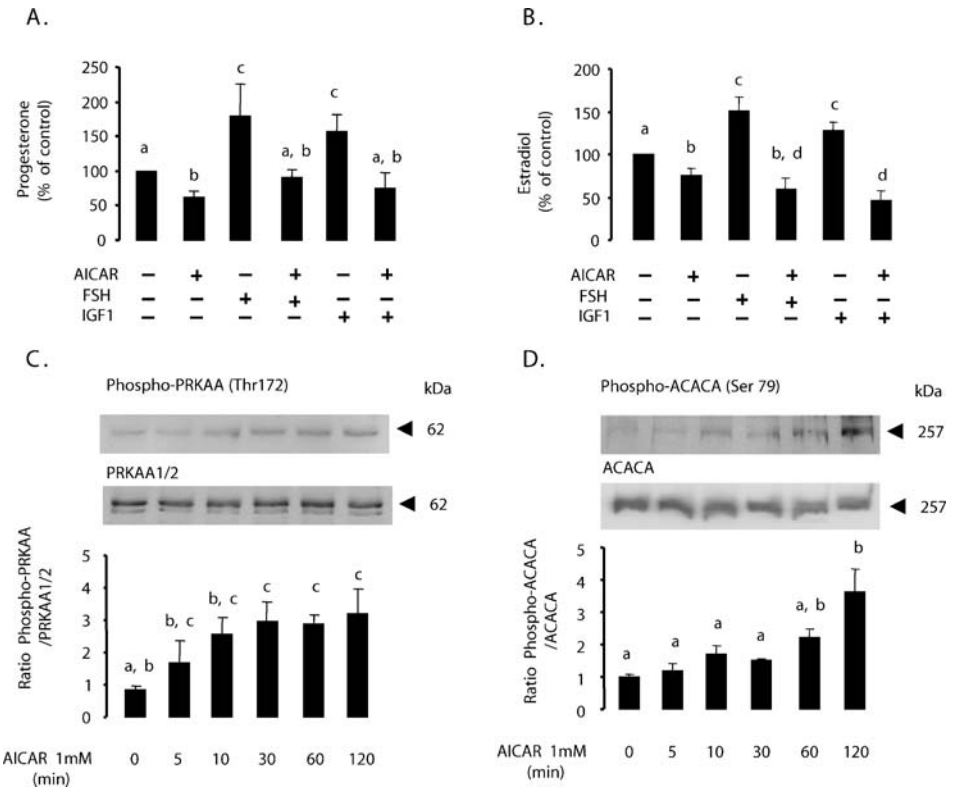

FIG. 5. Effect of AICAR treatment on basal and FSH- or IGF1-stimulated secretion of progesterone and estradiol (**A** and **B**) and on the phosphorylation of PRKAA and ACACA in bovine granulosa cells (**C** and **D**). Granulosa cells from small bovine follicles were cultured for 48h in medium with serum and then in serum-free medium in the presence or in the absence of 1mM AICAR ± 10^{-8} M FSH or ± 10^{-8} M IGF1 as described in *Materials and Methods*. The culture medium was then collected and analyzed for progesterone (**A**) and estradiol (**B**) content by RIA. Results are means ± SEM of three independent experiments. Bars with different letters are significantly different ($P < 0.05$). **C** and **D**) Granulosa cell lysates were prepared from cells incubated with 1mM AICAR for various times: 0, 5, 10, 30, 60, or 120 min. Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-PRKAA and anti-PRKAA1/2 (**C**) or anti-phospho-ACACA and anti-ACACA (**D**) antibodies. Representative blots from three independent experiments are shown. Bands on the blots were quantified and the phosphorylated protein / total protein ratio is shown. The results are reported as means ± SEM. Bars with different letters are significantly different ($P < 0.05$).

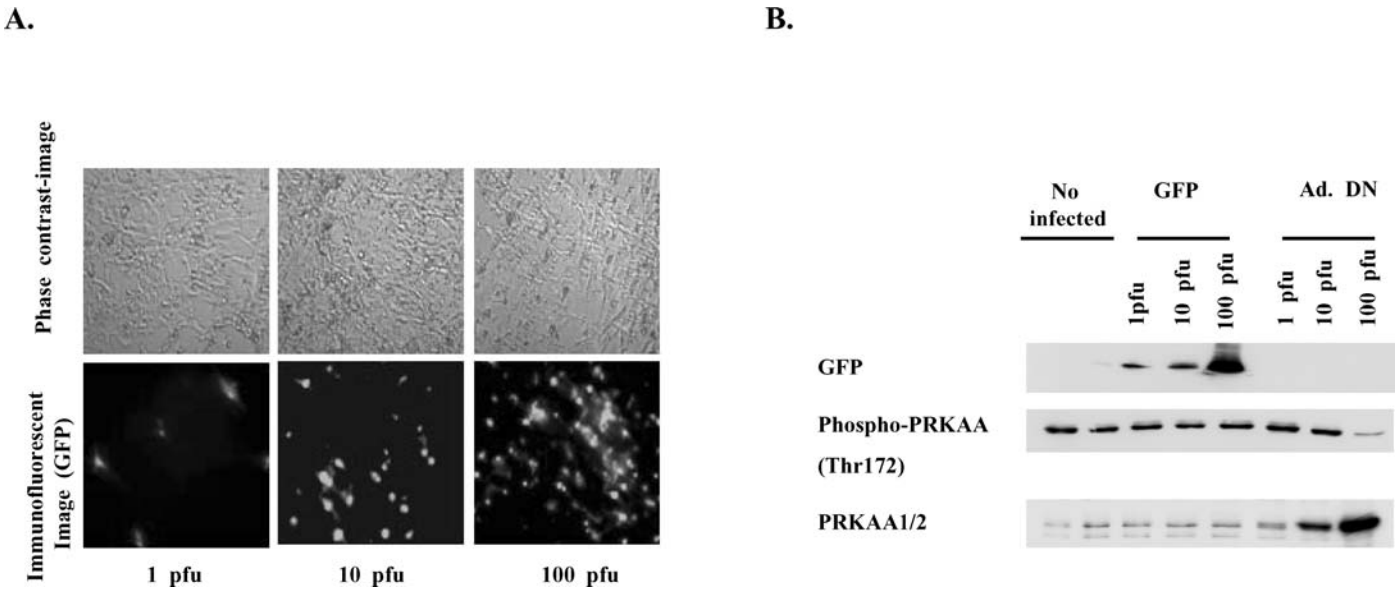

FIG. 6. Expression of mutant PRKAA1 adenovirus (Ad.DN) or GFP adenovirus (Ad.GFP) in primary bovine granulose cells. **A**) The upper panels show phase-contrast images of granulosa cells from small follicles infected with 1, 10, or 100 pfu/cell of the GFP adenovirus for 24 h cells. The lower panels show immunofluorescent images of cells expressing the GFP construct. **B**) Level of PRKAA phosphorylation in bovine granulosa cells infected with adenovirus constructs expressing a mutant PRKAA1 construct (Ad.DN) or a GFP construct (Ad.GFP). Granulosa cells were infected with 1, 10, or 100 Pfu/cell of virus as indicated in the *Materials and Methods*. After 16 h, cell lysates were prepared and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membranes, and probed with anti-phospho-PRKAA Thr172, anti-PRKAA1/2 and anti-GFP. The immunoblots shown are representative of three independent experiments.

Progesterone and Estradiol Radioimmunoassay

The concentration of progesterone and estradiol in the culture medium of granulosa cells was measured after 48 h of culture by a radioimmunoassay protocol as previously described [24]. The limit of detection of progesterone was 12 pg/tube (60 pg/well) and the intra- and interassay coefficients of variation were less than 10% and 11%, respectively. The limit of detection of estradiol was 1.5 pg/tube (7.5 pg/well) and the intra- and interassay coefficients of variation were less than 7% and 9%, respectively. Results are expressed as the amount of steroid (ng/ml) secreted over 48 h per 100 μg of protein.

Statistical Analysis

All experimental data are presented as means ± SEM. One-way analysis of variance was used to test differences except for the data reported in Figures 1C and D for which a two-way analysis of variance were used. $P < 0.05$ was considered to be statistically significant. Statview software was used for all statistical tests.

RESULTS

Effects of Metformin Treatment on Basal and FSH or IGF1-Stimulated Progesterone and Estradiol Productions in Granulosa Cells from Bovine Small Follicles

To investigate the effect of metformin treatment on the production of progesterone and estradiol, bovine granulosa cells from small follicles were incubated with various concentrations of metformin (0, 0.1, 0.5, 1, 5, and 10 mM) for 48 h, with 10 mM metformin for various times (0 h, 3 h, 6 h, 12 h, 24 h, and 48 h) or with 10 mM metformin for 48 h in the presence or absence of FSH (10^{-8} M) or IGF1 (10^{-8} M). Secretion of both progesterone (Figure 1A) and estradiol (Figure 1B) were inhibited by metformin treatment (48h) in a dose-dependent manner ($P < 0.001$). Metformin treatment (10 mM) significantly ($P < 0.05$) reduced progesterone and estradiol productions after 12 h, 24 h, and 48 h of incubation (Fig. 1, C and D). Two-way ANOVA indicates a significant effect of the duration of stimulation, a significant effect of the metformin treatment, and a significant interaction between the duration of stimulation and the metformin treatment for secretion of each progesterone (Fig. 1C) and estradiol (Fig. 1D). We have also determined whether metformin treatment affected the FSH and IGF1-induced production of progesterone and estradiol. In the presence of FSH (10^{-8} M) or IGF1 (10^{-8} M), metformin (10 mM, 48 h) decreased progesterone secretion by almost twofold ($P < 0.001$) (Fig. 1E). Metformin reduced estradiol production by almost twofold in the presence of FSH ($P < 0.05$) and threefold ($P < 0.001$) in that of IGF1 (Fig. 1F). Thus, metformin decreased both basal and FSH or IGF1-stimulated progesterone and estradiol production in granulosa cells from bovine small follicles.

We next examined whether the inhibitory effect of metformin on progesterone production was due to effects on the two key enzymes of steroidogenesis (HSD3B and CYP11A1) and/or of STAR, an important cholesterol carrier. Metformin treatment (10 mM, 48 h) in absence or presence of FSH or IGF1 halved the production of the HSD3B (Fig. 2A), CYP11A1 (Fig. 2B), and STAR proteins (Fig. 2C). Thus, the decrease in progesterone and estradiol secretion in response to metformin treatment may be due to a reduction in the amounts of the HSD3B, CYP11A1, and STAR proteins.

Characterization of AMPK in Bovine Ovary

We next identified the AMPK subunits in the bovine ovary. RT-PCR analysis with RNA from bovine ovary, dissected large and small follicles, corpus luteum and immature oocytes, resulted in the amplification of seven cDNAs. These cDNAs corresponded to fragments of two different isoforms of the catalytic α-subunit, *PRKAA1* (500 bp) and *PRKAA2* (445 bp), two different isoforms of the regulatory β-subunit, *PRKAB1* (460 bp) and *PRKAB2* (360 bp), and three different isoforms of the regulatory γ-subunit, *PRKAG1* (600 bp), *PRKAG2* (620 bp), and *PRKAG3* (300 bp) of AMPK (Fig. 3A). Immunoblotting protein extracts revealed one band corresponding to

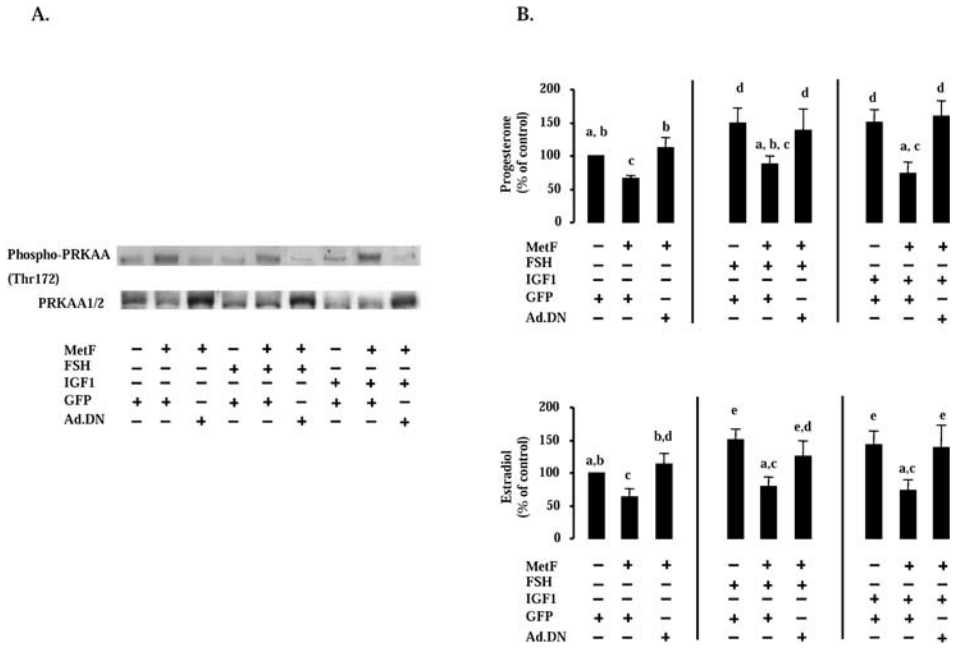

FIG. 7. Metformin-induced decreases in the secretion of progesterone and estradiol by bovine granulosa cells infected with adenovirus expressing PRKAA1 mutant constructs. **A**) Production and phosphorylation of PRKAA after infection of bovine granulosa cells with an adenovirus expressing either a PRKAA1 mutant construct or the GFP protein. Granulosa cells were infected with 100 Pfu/cell of either Ad.GFP or Ad.DN for 16h and were then stimulated or not stimulated for 36 h with 10 mM metformin ± 10^{-8} M FSH or ± 10^{-8} M IGF1. Cell lysates were prepared and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membranes, and probed with anti-phospho-PRKAA Thr172 and anti-PRKAA1/2. The immunoblots shown are representative of three independent experiments. **B**) Secretion of progesterone (upper panel) and estradiol (lower panel) by granulosa cells producing the mutant dominant negative PRKAA1 or the GFP protein in the presence or absence of 10 mM metformin ± 10^{-8} M FSH or ± 10^{-8} M IGF1. Granulosa cells were infected with 100 Pfu/cell of either Ad.GFP or Ad.DN for 16 h and were then stimulated or not stimulated for 36h with 10 mM metformin ± 10^{-8} M FSH or ± 10^{-8} M IGF1. The culture medium was then collected and its progesterone and estradiol content was analyzed by RIA. The data shown are means ± SEM from three independent experiments for the concentrations of progesterone and estradiol. Data are expressed as percentages of the value for unstimulated GFP-infected cells. Bars with different letters are significantly different ($P < 0.05$).

PRKAA1/2 (62 kDa) and two bands corresponding to PRKAB1 (40 kDa) and PRKAB2 (34 kDa) (Fig. 3B), showing that both subunits α and β of AMPK are produced in bovine ovary, and in particular in fresh granulosa cells from small and large follicles and in whole small and large follicles. Immunohistochemistry with bovine ovarian sections confirmed the presence of the PRKAA1 in bovine follicles (Fig. 3C). PRKAA1 was detected in the oocyte and its surrounding cells in primary follicles (panel a), in granulosa and theca cells (panels c and e), and in cumulus cells and oocyte from antral follicles (panels g and h). Thus, AMPK is present in the main structures of the bovine ovary.

Effects of Metformin Treatment on the Phosphorylation of PRKAA and ACACA in Granulosa Cells from Bovine Small Follicles

Metformin treatment activates PRKAA in various cell types including rat granulosa cells [13, 24]. We analyzed the pattern of PRKAA phosphorylation in bovine granulosa cells following various times of metformin (10 mM) treatment (0, 5, 10, 30, 60, and 120 min) and after 120 min of treatment with various doses of metformin (0, 0.1, 0.5, 1, 5, and 10 mM). We used an anti-phospho-PRKAA Thr172 antibody, specific for phosphorylated catalytic α-subunit. Metformin treatment (10 mM) increased PRKAA phosphorylation after 60 and 120 min of incubation (Fig. 4A, $P < 0.05$) and in a dose-dependent manner after 120 min of stimulation (Fig. 4B, $P < 0.05$). We also indirectly assessed the AMPK activity by measuring the phosphorylation of its downstream target, ACACA. Metformin increased the phosphorylation of ACACA on Ser79 in a time-dependent (Fig. 4C, $P < 0.05$) and dose-dependent manner (Fig. 4D, $P < 0.05$); this phosphorylation paralleled that of Thr172 PRKAA (Fig. 4B). Thus, metformin is able to activate PRKAA in cultured bovine granulosa cells. We also determined the effect of the serum starvation on the PRKAA phosphorylation. As shown in supplemental Figure 1 (available online at www.biolreprod.org), serum starvation for 24 or 48 h significantly increased phosphorylation of PRKAA in bovine granulosa cells cultured for 48 h with medium with serum. However, this effect was about half of that observed in response to metformin (10 mM, 48 h).

Effects of AICAR Treatment on Basal and FSH or IGF1-Stimulated Progesterone and Estradiol Production and on the Phosphorylation of PRKAA and ACACA in Bovine Granulosa Cells

We used 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), a pharmacological activator of AMPK [22], to determine the effects of AMPK activation on steroidogenesis in bovine granulosa cells. Granulosa cells were incubated for 48 h with 1 mM AICAR in the presence or absence of FSH (10^{-8} M) or IGF1 (10^{-8} M). Secretion of progesterone (Fig. 5A) and estradiol (Fig. 5B) were significantly inhibited by AICAR treatment in both the basal state and under FSH or IGF1 stimulation. Treatment with 1mM AICAR increased the phosphorylation of Thr172 of PRKAA and those of ACACA (Fig. 5, C and D, $P < 0.05$). AICAR treatment (1mM) therefore activates AMPK and inhibits both basal and FSH- or IGF1-stimulated progesterone and estradiol production in

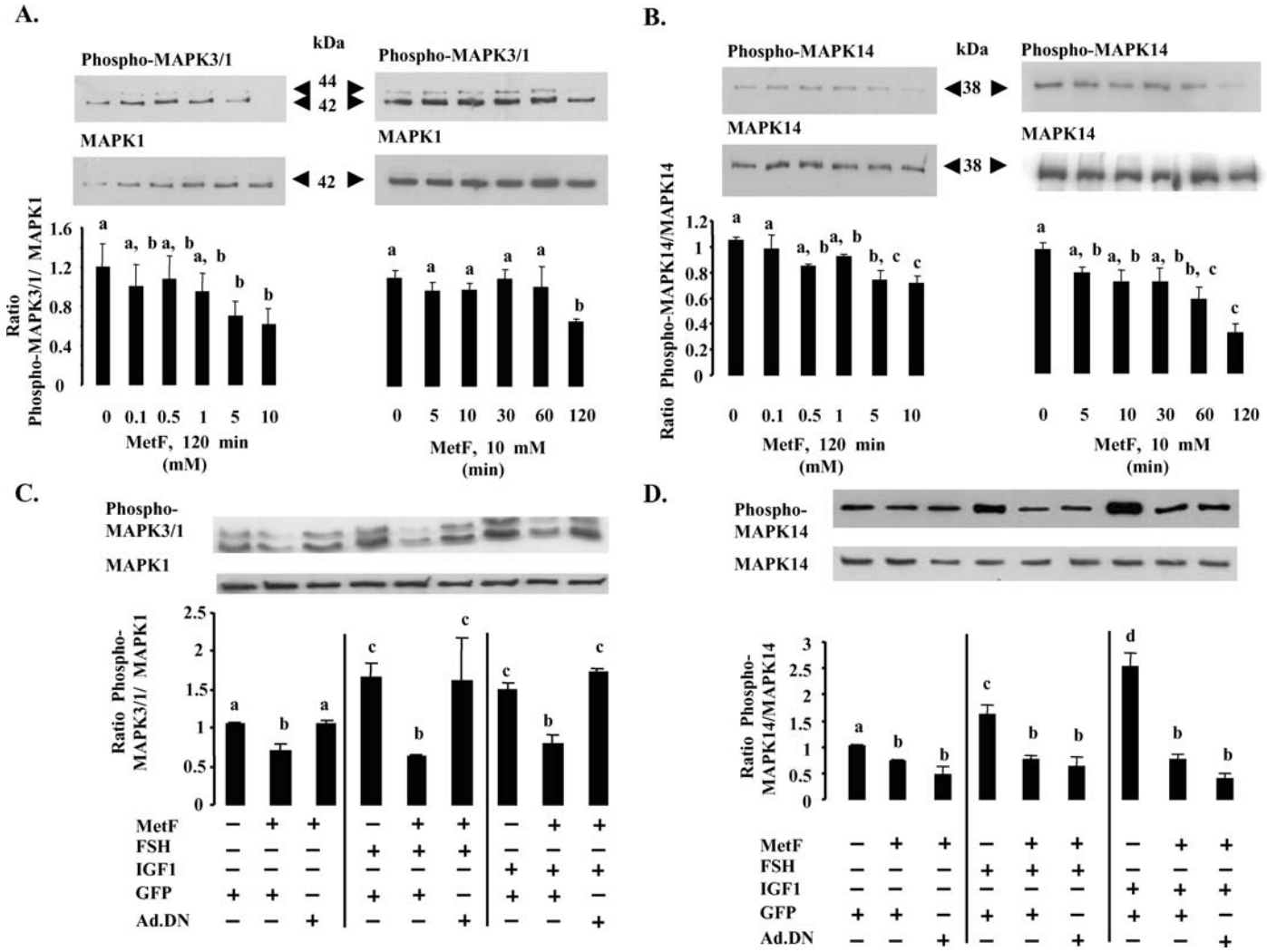

FIG. 8. Effect of metformin treatment on basal and FSH- or IGF1-stimulated phosphorylation of MAPK3/MAPK1 and MAPK14 and the potential involvement of AMPK in bovine granulosa cells. **A** and **B**) Granulosa cells from small bovine follicles were cultured for 48 h in medium with serum and then in serum-free medium in the presence or in the absence of various doses of metformin for 120 min (left panel), or for various times with 10 mM metformin (right panel). Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-MAPK3/MAPK1 and anti-MAPK1 (A) or anti-phospho-MAPK14 and anti-MAPK14 (B) antibodies. Representative blots from three independent experiments are shown. Bands on the blots were quantified and the phosphorylated protein / total protein ratio is shown. The results are reported as means ± SEM. Bars with different letters are significantly different ($P < 0.05$). **C** and **D**) Level of MAPK3/1 and MAPK14 phosphorylation in bovine granulosa cells infected with adenovirus constructs expressing a mutant PRKAA1 construct (Ad.DN) or a GFP construct (Ad.GFP). Granulosa cells were infected with 100 Pfu/cell of virus for 16h and then incubated with metformin (10 mM, 120 min) and then stimulated or not stimulated with FSH (10^{-8}M, 30 min) or IGF1 (10^{-8}M, 30 min). Cell lysates were then prepared and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membranes, and probed with anti-phospho-MAPK3/1, anti-MAPK1 and anti-phospho-MAPK14 and MAPK14. The immunoblots shown are representative of three independent experiments. Bands on the blots were quantified and the phosphorylated protein/total protein ratio is shown. The results are reported as means ± SEM. Bars with different letters are significantly different ($P < 0.05$).

bovine granulosa cells. Thus, these effects are similar to those described in response to metformin treatment.

Effects of the Overexpression of Dominant Negative PRKAA1 (Ad.DN) on Metformin-Inhibited Production of Progesterone and Estradiol in Bovine Granulosa Cells

We next investigated whether the metformin-induced decrease in the production of progesterone and estradiol was mediated by AMPK. We used an adenoviral vector (Ad.DN) to overexpress a dominant-negative PRKAA1 in bovine granulosa cells and treated these cells with FSH (10^{-8} M) or IGF1 (10^{-8} M) and metformin (10 mM) during 36h. Granulosa cells were infected with various amounts of Ad.DN and Ad.GFP (Ad. GFP as a control) adenovirus (1, 10, and 100 pfu) and were analyzed by Western blotting for production of mutant and endogenous PRKAA subunits (Fig. 6, A and B). Ad.DN proteins (with 100 pfu) were produced abundantly (Figs. 6B and 7A) and they significantly attenuated basal PRKAA Thr172 phosphorylation without affecting cell viability (data not shown). Cell infection with control (Ad.GFP, 100 pfu) had no effect on PRKAA expression or PRKAA Thr172 phosphorylation (Figs. 6B and 7A). Expression of Ad.DN in bovine granulosa cells strongly reduced the metformin-induced decrease in the production of progesterone and estradiol in both the presence and absence of FSH and IGF1 (Fig. 7B, upper and lower panel). The expression of Ad.GFP in the same conditions did not restore these inhibitory effects of metformin (Fig. 7B, upper and lower panel). Thus, metformin reduces

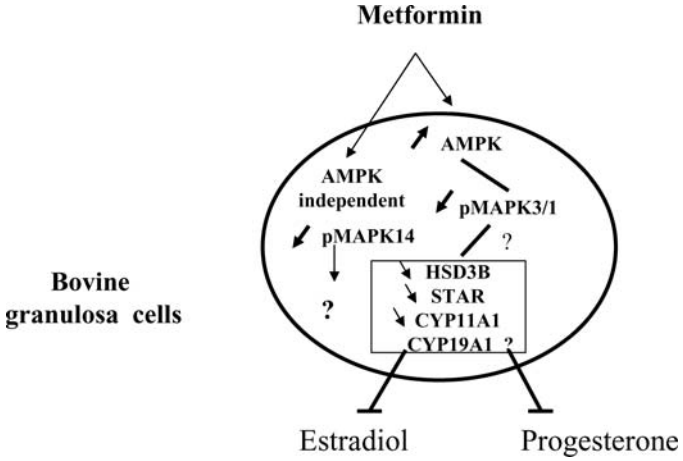

FIG. 9. Schematic representation of the effects of metformin on granulosa cells from bovine small follicles. In bovine granulosa cells, metformin-induced PRKAA activation decreases secretion of progesterone and estradiol and phosphorylation of MAPK3/MAPK1 in the basal state and in response to IGF1 and FSH. The decrease in progesterone secretion appears to be due to reduction in the amounts of STAR, HSD3B, and CYP11A1 proteins. The involvement, if any, of the CYP19A1 protein in the metformin-induced decrease in estradiol production remains to be determined. Metformin also inhibits MAPK14 phosphorylation through a PRKAA-independent pathway. The biological effects of metformin-induced MAPK14 phosphorylation are unknown.

progesterone and estradiol secretion through activation of PRKAA in bovine granulosa cells.

Signaling Pathways Involved in the Metformin Effects in Bovine Granulosa Cells

Metformin significantly inhibited phosphorylation of MAPK3/MAPK1 and MAPK14 in a dose- and time-dependent manner in bovine granulosa cells (Fig. 8A and B). Under the same conditions, phosphorylation of AKT was unchanged (data not shown). Expression of Ad.DN in bovine granulosa cells totally abolished the metformin-induced decrease of MAPK3/MAPK1 phosphorylation in both the presence and absence of FSH or IGF1 (Fig. 8C) whereas it did not affect the metformin-induced inhibition of MAPK14 phosphorylation in the same conditions (Fig. 8D). The expression of Ad.GFP did not restore these inhibitory effects of metformin (Fig. 8, C and D). Thus, metformin-induced PRKAA activation inhibits MAPK3/MAPK1 phosphorylation in bovine granulosa cells.

Effects of Metformin and AICAR Treatments on Granulosa Cell Proliferation and Viability

We also investigated whether or not the dose of metformin and AICAR used (10 mM and 1 mM, respectively) affected the number of granulosa cells in culture, either by induction of mitosis or by altering the cell viability. [^{3}H]-thymidine incorporation by granulosa cells was determined after 24 h of culture in the presence or in the absence of FSH (10^{-8} M) or IGF1 (10^{-8} M). FSH treatment significantly increased [^{3}H]-thymidine incorporation by about 20% ($P < 0.05$). IGF1 had a very small effect (about 10%) on cell proliferation probably due to the presence of insulin (50 μg/l) in the culture medium. Metformin and AICAR treatments slightly but significantly decreased both basal and FSH or IGF1-stimulated [^{3}H]-thymidine incorporation by about 5%–20% (supplemental Fig. 2, available online at www.biolreprod.org). Staining with trypan blue revealed that metformin and AICAR treatments had no effect on cell viability in presence or absence of FSH or IGF1 (data not shown). Thus, metformin decreases cell proliferation slightly but not sufficiently to explain the decrease in progesterone and estradiol production in response to metformin.

DISCUSSION

We report that metformin inhibits secretion of both progesterone and estradiol from cultured bovine granulosa cells through a PRKAA-dependent mechanism in both basal and stimulated (by FSH or IGF1) conditions (Fig. 9A). We also demonstrated that metformin reduces phosphorylation of both MAPK3/1 and MAPK14. However, only the metformin-induced decrease in MAPK3/1 appears to be dependent on PRKAA activation (Fig. 9A). We identified the various subunits of AMPK in several ovarian structures, in particular the corpus luteum and small and large follicles. In follicles, AMPK subunits are present in granulosa and theca cells and in oocytes.

We chose to study bovine granulosa cells from small follicles and not large follicles because we wanted to determine the effect of metformin on the cell proliferation induced by IGF1 or FSH. Granulosa cells from large follicles proliferate less than those from small follicles [33]. In a good agreement with some previous studies, we observed a strong inhibitory effect of metformin on steroidogenesis in granulosa cells from small bovine follicles. Indeed, metformin treatment decreased progesterone and estradiol productions in vitro by human [34] and rat [13] granulosa cells and in vivo in serum of women with polycystic ovary syndrome (PCOS) [11, 35, 36]. The dose of metformin (10 mM) we used is about 100 times those used in clinical protocols.

However, we also observed that metformin (100 μM) significantly decreased steroid secretions by bovine granulosa cells. As previsously shown in rat granulosa cells, the decrease in the production of progesterone and estradiol could be explained by metformin reducing the amount of some steroidogenic factors, including HSD3B, STAR, and CP11A1 in bovine granulosa cells [13]. Furthermore, the decrease in estradiol secretion in response to metformin could be due to a reduction in the amount of CYP19A1 protein, as reported in rat granulosa cells [13], or due to a decrease in CYP19A1 activity, as suggested for women with PCOS [11, 36]. Using an adenovivus PRKAA dominant negative, we showed that the inhibitory effects of metformin on both progesterone and estradiol were PRKAA-dependent. In rat granulosa cells, we found that the metformin-induced decrease in estradiol secretion was PRKAA-independent [13]. These observations suggest that metformin acts on estradiol production in granulosa cells through different molecular mechanisms according to the species. The control of estradiol production and the expression of CYP19A1 differ between rats and ruminants. For example, estradiol secretion and CYP19A1 gene expression occur in luteinized granulosa cells and copora lutea in rats [37, 38] but not in cattle [39, 40].

Here we showed that metformin reduces steroidogenesis in bovine granulosa cells from healthy small follicles. Thus, metformin may also reduce steroid levels in granulosa cells from follicular cysts. The formation of ovarian follicular cysts is one of the most important ovarian disorders affecting high lactating dairy cows [41, 42]. It contributes to reducing reproductive efficiency by increasing the calving interval by about 22–64 days [43]. Cystic ovarian follicles appear when one or more follicles fail to ovulate and consequently do not

regress but maintain growth and steroidogenesis [41]. Steroid secretion in the ovary of cystic cows is abnormal: for example, Hatler et al. observed that some ovarian cysts are accompanied by suprabasal progesterone concentrations [44]; and Calder et al. showed that estrogen-active cysts express high levels of HSD3B [45]. Moreover, cows developing an ovarian cyst have increased estradiol concentrations during the early stages of follicular dominance [46].

Cows with cystic ovarian follicles also suffer insulin insufficiency [47]. In humans, metformin treatment increases insulin sensitivity in various tissues. In our preliminary studies, we observed that metformin (10 mM) increases insulin-induced tyrosine phosphorylation of insulin receptor or IRS-1 (Insulin receptor substrate-1) in bovine granulosa cells (data not shown). However, further experiments in vitro and in vivo are necessary to judge to the value of metformin for the treatment of the ovarian follicular cyst in high dairy cows.

We found that metformin reduces phosphorylation of MAPK3/MAPK1 and MAPK14. These results agree with studies showing that metformin inhibited phospho-MAPK3/MAPK1 in various cell types including rat granulosa cells [13, 48]. Here, we show that the inhibition of MAPK3/MAPK1 phosphorylation in response to brief metformin stimulation seems to be dependent on the activation of PRKAA in bovine granulosa cells. As the MAPK3/MAPK1 pathway is directly involved in progesterone secretion in rat granulosa cells [13, 24], PRKAA activation may decrease progesterone and/or estradiol secretion by inhibiting this pathway in bovine granulosa cells. In bovine theca cells, the MAPK3/MAPK1 signaling pathway also appears to contribute to the control of steroidogenesis [49]. We showed that metformin decreases MAPK14 phosphorylation independently of AMPK activation.

Indeed, not all the effects of metformin are mediated through AMPK. For example, in rat hepatocytes, metformin activates PRKAA but inhibits hepatic glucose phosphorylation by a AMPK-independent mechanism [50]. The MAPK14 signalling pathway in bovine granulosa cells has never been studied. In rat granulosa cells, this pathway may be involved in cytoskeletal reorganization and cell survival [51] and also in steroidogenesis [52].

All AMPK subunit mRNAs were amplified by RT-PCR from samples of bovine whole ovaries, corpus luteum, and small and large follicles and oocytes. The presence of the PRKAG3 subunit mRNA was surprising because it has been described as muscle specific in rodent [53] and human [54]. The PRKAA1 subunit is expressed in theca, granulosa, and cumulus cells and oocytes of antral follicles. In mouse oocytes, activation of PRKAA in response to AICAR promoted resumption of meiosis arrested by cAMP analogues, phosphodiesterase inhibitors, or hypoxanthine [26]. Also, in pigs, metformin enhances insulin action on the developmental potential of oocytes and embryos [12]. We are currently investigating the effects of metformin and the involvement of AMPK on bovine oocyte maturation in vitro. AMPK activation has been described as having numerous roles in various cells including positive effects on glucose transport and fatty acid oxidation, and negative effects on the synthesis of cholesterol, glycogen, and fatty acid [55]. Thus, metformin, though AMPK activation, could affect functions in addition to steroidogenesis in bovine granulosa cells.

In conclusion, metformin decreases the production of both progesterone and estradiol in cultured granulosa cells from small follicles through activation of PRKAA and possibly inhibition of the MAPK3/MAPK1 signalling pathway. Further experiments are required in vitro with granulosa cells from bovine cystic follicles and in vivo in whole animals to assess the value of using metformin to treat infertility disorders in high lactating dairy cows.

ACKNOWLEDGMENTS

We thank Pascal Ferré and Fabienne Foufelle for the PRKAA dominant negative adenovirus.

REFERENCES

1. Spicer LJ, Chamberlain CS, Maciel SM. Influence of gonadotropins on insulin-and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. Domest Anim Endocrinol 2002; 22:237–254.
2. Dominguez LJ, Davidoff AJ, Srinivas PR, Standley PR, Walsh MF, Sowers JR. Effects of metformin on tyrosine kinase activity, glucose transport, and intracellular calcium in rat vascular smooth muscle. Endocrinology 1996; 137:113–121.
3. van Santbrink EJ, Hohmann FP, Eijkemans MJ, Laven JS, Fauser BC. Does metformin modify ovarian responsiveness during exogenous FSH ovulation induction in normogonadotrophic anovulation? A placebo-controlled double-blind assessment. Eur J Endocrinol 152; 4:611–617.
4. Howlett HC, Bailey CJ. A risk-benefit assessment of metformin in type 2 diabetes mellitus. Drug Saf 1999; 20:489–503.
5. Velazquez E, Acosta A, Mendoza SG. Menstrual cyclicity after metformin therapy in polycystic ovary syndrome. Obstet Gynecol 1997; 90:392–395.
6. Vandermolen DT, Ratts VS, Evans WS, Stovall DW, Kauma SW, Nestler JE. Metformin increases the ovulatory rate and pregnancy rate from clomiphene citrate in patients with polycystic ovary syndrome who are resistant to clomiphene citrate alone. Fertil Steril 2001; 75:310–315.
7. Bailey CJ. Metformin and its role in the management of type II diabetes. Curr Opin Endocrinol Diabet 1997; 4:40–47.
8. Nardo LG, Rai R. Metformin therapy in the management of polycystic ovary syndrome: endocrine, metabolic and reproductive effects. Gynecol Endocrinol 2001; 15:373–380.
9. Attia GR, Rainey WE, Carr BR. Metformin directly inhibits androgen production in human thecal cells. Fertil Steril 2001; 76:517–524.
10. Nestler JE, Jakubowicz DJ. Lean women with polycystic ovary syndrome respond to insulin reduction with decreases in ovarian P450c17 alpha activity and serum androgens. J Clin Endocrinol Metab 1997; 82:4075–4079.
11. La Marca A, Morgante G, Palumbo M, Cianci A, Petraglia F, De Leo V. Insulin-lowering treatment reduces aromatase activity in response to follicle-stimulating hormone in women with polycystic ovary syndrome. Fertil Steril 2002; 78:1234–1239.
12. Lee MS, Kang SK, Lee BC, Hwang WS. The beneficial effects of insulin and metformin on in vitro developmental potential of porcine oocytes and embryos. Biol Reprod 2005; 73:1264–1268.
13. Tosca L, Solnais P, Ferre P, Foufelle F, Dupont J. Metformin-induced stimulation of AMP-activated protein kinase (PRKA) impairs progesterone secretion in rat granulosa cells. Biol Reprod 2006; 75:342–351.
14. Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 2005; 1:15–25.
15. Hardie DG. The AMP-activated protein kinase pathway—new players upstream and downstream. J Cell Sci 2004; 117:5479–5487.
16. Birnbaum MJ. Activating AMP-activated protein kinase without AMP. Mol Cell 2005; 19:289–290.
17. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ. Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. Diabetes 1998; 47:1369–1373.
18. Mu J, Brozinick JT Jr, Valladares O, Bucan M, Birnbaum MJ. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. Mol Cell 2001; 7:1085–1094.
19. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002; 8:1288–1295.
20. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature 2002; 415:339–343.
21. Fryer LG, Parbu-Patel A, Carling D. The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. J Biol Chem 2002; 277:25226–25232.
22. Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-

carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem 1995; 229:558–565.
23. Hardie DG, Carling D. The AMP-activated protein kinase—fuel gauge of the mammalian cell? Eur J Biochem 1997; 246:259–273.
24. Tosca L, Froment P, Solnais P, Ferre P, Foufelle F, Dupont J. Adenosine 5′-monophosphate-activated protein kinase regulates progesterone secretion in rat granulosa cells. Endocrinology 2005; 146:4500–4513.
25. Chen J, Hudson E, Chi MM, Chang AS, Moley KH, Hardie DG, Downs SM. AMPK regulation of mouse oocyte meiotic resumption in vitro. Dev Biol 2006; 291:227–238.
26. Downs SM, Hudson ER, Hardie DG. A potential role for AMP-activated protein kinase in meiotic induction in mouse oocytes. Dev Biol. 2002; 245:200–212.
27. LaRosa C, Downs SM. Stress stimulates AMP-activated protein kinase and meiotic resumption in mouse oocytes. Biol Reprod 2006; 74:585–592.
28. Downs SM, Chen J. Induction of meiotic maturation in mouse oocytes by adenosine analogs. Mol Reprod Dev 2006; 73:1159–1168.
29. McKay SD, White SN, Kata SR, Loan R, Womack JE. The bovine 5′ AMPK gene family: mapping and single nucleotide polymorphism detection. Mamm Genome 2003; 14:853–858.
30. Roux M, Nizou A, Forestier L, Ouali A, Leveziel H, Amarger V. Characterization of the bovine PRKAG3 gene: structure, polymorphism, and alternative transcripts. Mamm Genome 2006; 17:83–92.
31. Benkel B, Kollers S, Fries R, Sazanov A, Yoshida E, Valle E, Davoren J, Hickey D. Characterization of the bovine ampkgamma1 gene. Mamm Genome 2005; 16:194–200.
32. Woods A, Azzout-Marniche D, Foretz M, Stein SC, Lemarchand P, Ferre P, Foufelle F, Carling D. Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. Mol Cell Biol 2000; 20:6704–6011.
33. Basini G, Baratta M, Bussolati S, Tamanini C. Interleukin-1 beta fragment (163–171) modulates bovine granulosa cell proliferation in vitro: dependence on size of follicle. J Reprod Immunol 1998; 37:139–153.
34. Mansfield R, Galea R, Brincat M, Hole D, Mason H. Metformin has direct effects on human ovarian steroidogenesis. Fertil Steril 2003; 79: 956–962.
35. Eagleson CA, Bellows AB, Hu K, Gingrich MB, Marshall JC. Obese patients with polycystic ovary syndrome: evidence that metformin does not restore sensitivity of the gonadotropin-releasing hormone pulse generator to inhibition by ovarian steroids. J Clin Endocrinol Metab 2003; 88:5158–5162.
36. Vrbikova J, Hill M, Starka L, Cibula D, Bendlova B, Vondra K, Sulcova J, Snajderova M. The effects of long-term metformin treatment on adrenal and ovarian steroidogenesis in women with polycystic ovary syndrome. Eur J Endocrinol 2001; 144:619–628.
37. Hickey GJ, Krasnow JS, Beattie WG, Richards JS. Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3′,5′-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5′ genomic DNA. Mol Endocrinol 1990; 4:3–12.
38. Gonzalez-Robayna IJ, Alliston TN, Buse P, Firestone GL, Richards JS. Functional and subcellular changes in the A-kinase-signaling pathway: relation to aromatase and Sgk expression during the transition of granulosa cells to luteal cells. Mol Endocrinol 1999; 13:1318–1337.
39. Hinshelwood MM, Corbin CJ, Tsang PC, Simpson ER. Isolation and characterization of a complementary deoxyribonucleic acid insert encoding bovine aromatase cytochrome P450. Endocrinology 1993; 133: 1971–1977.
40. Gutierrez CG, Campbell BK, Webb R. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. Biol Reprod 1997; 56:608–616.
41. Garverick HA. Ovarian follicular cysts in dairy cows. J Dairy Sci 1997; 80:995–1004.
42. Vanholder T, Opsomer G, de Kruif A. Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. Reprod Nutr Dev 2006; 46:105–119.
43. Borsberry S, Dobson H. Periparturient diseases and their effect on reproductive performance in five dairy herds. Vet Rec 1989; 124:217–219.
44. Hatler TB, Hayes SH, Laranja da Fonseca LF, Silvia WJ. Relationship between endogenous progesterone and follicular dynamics in lactating dairy cows with ovarian follicular cysts. Biol Reprod 2003; 69:218–223.
45. Calder MD, Manikkam M, Salfen BE, Youngquist RS, Lubahn DB, Lamberson WR, Garverick HA. Dominant bovine ovarian follicular cysts express increased levels of messenger RNAs for luteinizing hormone receptor and 3 beta-hydroxysteroid dehydrogenase delta(4), delta(5) isomerase compared to normal dominant follicles. Biol Reprod 2001; 65:471–476.
46. Beam SW. Follicular development in post-partum cattle: effects of energy balance and dietary lipid. PhD dissertation, Cornell University, 1995;124–136.
47. Opsomer G, Wensing T, Laevens H, Coryn M, de Kruif A. Insulin resistance: the link between metabolic disorders and cystic ovarian disease in high yielding dairy cows? Anim Reprod Sci 1999; 56:211–222.
48. Li L, Mamputu JC, Wiernsperger N, Renier G. Signaling pathways involved in human vascular smooth muscle cell proliferation and matrix metalloproteinase-2 expression induced by leptin: inhibitory effect of metformin. Diabetes 2005; 54:2227–2234.
49. Tajima K, Yoshii K, Fukuda S, Orisaka M, Miyamoto K, Amsterdam A, Kotsuji F. Luteinizing hormone-induced extracellular-signal regulated kinase activation differently modulates progesterone and and rostenedione production in bovine theca cells. Endocrinology 2005; 146:2903–2910.
50. Guigas B, Bertrand L, Taleux N, Foretz M, Wiernsperger N, Vertommen D, Andreelli F, Viollet B, Hue L. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase-independent effect on glucokinase translocation. Diabetes 2006; 55:865–874.
51. Maizels ET, Cottom J, Jones JC, Hunzicker-Dunn M. Follicle stimulating hormone (FSH) activates the p38 mitogen-activated protein kinase pathway, inducing small heat shock protein phosphorylation and cell rounding in immature rat ovarian granulosa cells. Endocrinology 1998; 139:3353–3356.
52. Yu FQ, Han CS, Yang W, Jin X, Hu ZY, Liu YX. Activation of the p38 MAPK pathway by follicle-stimulating hormone regulates steroidogenesis in granulosa cells differently. J Endocrinol 2005; 186:85–96.
53. Durante PE, Mustard KJ, Park SH, Winder WW, Hardie DG. Effects of endurance training on activity and expression of AMP-activated protein kinase isoforms in rat muscles. Am J Physiol Endocrinol Metab 2002; 283: E178–E186.
54. Nielsen JN, Mustard KJ, Graham DA, Yu H, MacDonald CS, Pilegaard H, Goodyear LJ, Hardie DG, Richter EA, Wojtaszewski JF. 5′-AMP-activated protein kinase activity and subunit expression in exercise-trained human skeletal muscle. J Appl Physiol 2003; 94:631–641.
55. Long YC, Zierath JR. AMP-activated protein kinase signalling in metabolic regulation. J Clin Invest 2006; 116:1776–1783.