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Induction of Alpha-Caveolin-1 (αCAV1) Expression in Bovine Granulosa Cells in Response to an Ovulatory Dose of Human Chorionic Gonadotropin

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ABSTRACT Caveolins are implicated in endocytosis, cholesterol trafficking and signal transduction. A cDNA fragment corresponding to caveolin-1 (*CAV1*) was identified in a mRNA profiling expression study in bovine granulosa cells (GC) following human chorionic gonadotropin (hCG)-induced ovulation. Thus, we have characterized *CAV1* cDNA and studied its spatio-temporal expression pattern in bovine ovarian follicles. The full-length bovine *αCAV1* cDNA was cloned and encodes a putative 22 kDa protein. Expression of αCAV1 was studied in bovine GC obtained from follicles at different developmental stages: small follicles (SF: 2–4 mm), dominant follicles (DF), ovulatory follicles (OF: 24 hr post-hCG), and corpus luteum (CL). Semiquantitative RT-PCR analysis showed a 6.5-fold increase in *αCAV1* mRNA in GC of OF versus DF ($P < 0.0001$), whereas *CAV2* mRNA was increased by only twofold ($P < 0.0007$). Temporal expression of *αCAV1* mRNA from OF recovered at 0, 6, 12, 18, and 24 hr after hCG injection showed an 8.5-fold increase of *αCAV1* mRNA after 24 hr compared to 0 hr ($P < 0.0018$) whereas no significant variation was detected for *CAV2*. Immunoblot demonstrated an initial increase in αCAV1 protein level 12 hr post-hCG, reaching a maximum at 24 hr. Immunohistochemical localization of CAV1 was observed in GC of OF isolated 18 and 24 hr after hCG injection, whereas no signal was detected in GC of DF and SF. The induction of αCAV1 in GC of OF suggests that αCAV1 likely contributes to control the increase in membrane signaling that occurs at the time of ovulation and luteinization. *Mol. Reprod. Dev. 73: 1353–1360, 2006.* © 2006 Wiley-Liss, Inc.

Key Words: ovary; follicle; granulosa cell; gene expression; ovulation; caveolin; CAV1; CAV2

INTRODUCTION

The ovulatory and luteinization processes in mammals begin at the time when the endogenous luteinizing hormone (LH) surge stimulates the G protein-coupled LH/human chorionic gonadotropin receptors (LHCGR) that are distributed on the surface of granulosa and theca cells of a mature preovulatory follicle. Gonadotropin-stimulated ovulation and luteinization are controlled by the induction (Robker et al., 2000; Espey and Richards, 2002; Lévesque et al., 2002; Jo et al., 2004) and the downregulation of specific genes (Fayad et al., 2004; Ndiaye et al., 2005) that are spatially and temporally regulated in the different follicular compartments during the periovulatory period. Based on a previous mRNA expression profiling study applying subtractive suppression hybridization (SSH), a cDNA fragment that corresponded to bovine *CAV1* was identified as a candidate gene that would be induced in Granulosa cells (GC) by the LH/hCG preovulatory surge (Lévesque et al., 2002). Thus, we hypothesized that expression of the CAV1 in GC of ovulatory follicles (OF) is induced by the LH/hCG preovulatory surge, which would contribute to ovulation and luteinization. The specific objectives of this study were to clone the corresponding full-length bovine *CAV1* cDNA, and to study the spatio-temporal expression profile of CAV1 mRNA and protein in bovine OF following hCG treatment.

MATERIALS AND METHODS

Cloning of Bovine Caveolin-1 cDNA

Isolation of the full-length bovine *CAV1* cDNA was performed by screening a size-selected cDNA library. Initially, the size of the full-length bovine *CAV1* cDNA was estimated by performing a virtual Northern blot analysis as described (Lévesque et al., 2003), using total RNA isolated from GC that were obtained from bovine OF 24 hr following an injection of an ovulatory dose of hCG (3000 IU; APL Ayerst Lab, Montréal, PQ) (Ndiaye et al., 2005). The cDNAs from hCG-stimulated GC were size fractionated by agarose gel electrophoresis, and cDNAs from 2.3 to 2.7 kb were purified to construct a size-selected cDNA library based on the pDrive plasmid

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(Qiagen PCR cloning kit; Qiagen, Mississauga, ON). The cDNA library was then screened by radioactive hybridization as described (Lévesque et al., 2003). Positive CAV1 hybridizing bacterial colonies were grown, their plasmid content was isolated (QIA-prep, Qiagen), and the size of the cloned cDNA was analyzed following *EcoR*1 digestion and gel electrophoresis analysis. The cDNAs were sequenced via the dideoxy sequencing method (Big Dye Terminator 3.0; ABI Prism, Applied BioSystem, PE, Branchburg, NJ) and analyzed on an ABI Prism 310 sequencer (Applied Biosystem). Nucleic acid sequences were analyzed by BLAST (Basic Local Alignment Search Tool) against GenBank data banks.

Bovine Tissues and RNA Extraction

Bovine ovarian follicles and corpora lutea (CL) were isolated at specific stages of the estrous cycle from normal cycling crossbred heifers, as described (Ndiaye et al., 2005). Briefly, estrous cycle was synchronized with $PGF_{2\alpha}$ (25 mg, im; Lutalyse, Upjohn, Kalamazoo, MI) and ovarian follicular development was monitored by daily transrectal ultrasonography until ovariectomy. Dominant follicles (DF; n = 4 cows) were defined by ultrasonographic measurement as >8 mm and growing while subordinate follicles were either static or regressing. DF were obtained on the morning of day 5 of the estrous cycle (day 0 = day of estrus) by ovariectomy. OF were obtained following an injection of 25 mg of $PGF_{2\alpha}$ on day 7 of the synchronized estrous cycle to induce luteolysis, thereby maintaining the development of the DF of the first follicular wave into a preovulatory follicle. An ovulatory dose of hCG (3000 IU, iv; APL, Ayerst Lab, Montréal, QC) was injected 36 hr after the induction of luteolysis, and the ovary bearing the hCG-induced OF was collected by ovariectomy at 0, 6, 12, 18, and 24 hr after hCG injection (n = 2–4 cows/time point). Follicles were dissected into preparations of follicular wall (theca interna with attached GC) (Filion et al., 2001) or further dissected into separate isolates of GC (Ndiaye et al., 2005). Ovariectomies were also performed on day 5 of the synchronized estrous cycle to obtain CL (n = 3 cows). Additionally, GC were collected from 2 to 4 mm follicles that were obtained from slaughterhouse ovaries representing a total of three pools of 20 small follicles (SF). These experiments were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine of the Université de Montréal. Total RNA was isolated from tissues as described (Bédard et al., 2003). The concentration of total RNA was quantified by measuring the optical density at 260 nm, and quality was evaluated by visualizing the 28S and 18S ribosomal bands following electrophoretic separation on a formaldehyde denaturing 1% agarose gel with ethidium bromide (Bédard et al., 2003).

Semiquantitative RT-PCR Analysis

Total RNA (1 μg) from individual follicles or CL was reverse-transcribed with an oligo-dT30 primer and PowerScript (BD Biosciences Clontech, Mississauga, ON) to generate the first strand cDNA, using the SMART PCR cDNA synthesis kit (BD Biosciences Clontech), and PCR-amplified for 18 cycles using Advantage 2 DNA polymerase (BD Biosciences Clontech) as described (Ndiaye et al., 2005). SMART cDNA pools were then diluted to 50 μl in 10 mM Tris pH 8 buffer. To perform semiquantitative RT-PCR, SMART cDNA pools were diluted 10-fold in H_2O, and 1–2 μl were used in a 25 μl PCR reaction using the advantage 2 DNA polymerase kit (BD Biosciences Clontech). Gene-specific PCR primers were designed in the open reading frame of the bovine cDNA sequence for: *CAV1* (sense: 5′-GGCAAATACGTAGACTCAGAGGGAC-3′; anti-sense: 5′-CTGCGTGTTGATGCGGATATTGCTG-3′; GenBank: AY823915), *CAV2* (sense: 5′-GATGTCCAGCTCTTCATGGACGAC-3′; anti-sense: 5′- GACGCAGCTTCT-CTTCTGTCAG-3′; GenBank: AY699947), and glyceraldehyde-3-phosphate dehydrogenase (*GAPD*; sense: 5′-TGTTCCAGTATGATTCCACCCACG-3′; anti-sense: 5′-CTGTTGAAGTCGCAGGAGACAACC-3′) (Ndiaye et al., 2005). For all these genes, PCR conditions were as follows: 95°C for 1 min, 95°C for 30 sec, 64°C for 45 sec, and 68°C for 90 sec. The number of PCR cycles was limited (14–21 cycles) and optimized for each gene to be analyzed. The PCR reactions (20 μl/reaction) were resolved on a 2% TAE-agarose gel (40 mM Tris acetate pH 8, 1 Mm EDTA) with ethidium bromide (0.5 μg/ml); PCR products were visualized by UV and the images were digitized. The digitized signals for each gene were analyzed by densitometry using ImageQuant software (Amersham Pharmacia Biotech, Pointe-Claire, QC).

Cell Extracts and Immunoblot Analysis

Tissues and cells were homogenized in M-PER buffer (Pierce, Rockford, IL) that was supplemented with a mix of protease inhibitors (Complete; Roche Applied Science, Laval, QC) as recommended by the manufacturer's protocol. Complete lysis of GC was achieved with multiple passages through a 25G needle attached to a 3-ml syringe. CL were homogeneized at 7,000 rpm with a polytron PT1300D (Kinematica AG, Littau-Lucerne, SW). The protein extracts were centrifuged at 16,000*g* for 15 min at 4°C, and the recovered supernatant (whole cell extract) was stored at −80°C. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA). Protein extracts (50 μg proteins/sample) were heat-treated (5 min, 100°C), size-fractionated via a one-dimensional 18% SDS–PAGE, and electrophoretically transferred onto polyvinylidene difluoride membranes (PVDF; Hybond-P, Amersham Pharmacia Biotech). Immunoblots were performed as described (Brûlé et al., 2003). Membranes were incubated with rabbit polyclonal antibody against human CAV1 (1:500 dilution; SC-894; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and detected with a horseradish peroxidase-linked sheep anti-rabbit secondary antibody (1:20,000 dilution; Amersham Pharmacia Biotech). Detection of immunoreactive proteins was performed by the enhanced chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech) following the manufacturer's protocol, and exposed to

Hyperfilm (Amersham Pharmacia Biotech). The expression of α-glutathione S-transferase-1 (GSTA1) was used as control (Rabahi et al., 1999). Membranes were incubated with a rabbit polyclonal antibody against bovine GSTA1 (1:3,000 dilution) and detected with an alkaline phosphatase-linked goat anti-rabbit secondary antibody (1:20,000 dilution; Sigma-Aldrich, Oakville, ON) using NBT/BCIP as alkaline phosphatase substrate (Roche Molecular Biochemicals). Images were digitized using a ScanMaker 9800XL flatbed scanner (Microtek lab, Inc., Redondo Beach, CA).

Immunohistochemical Localization of Caveolin-1

Immunohistochemical staining was performed on PBS-buffered formalin-fixed tissues as described (Brûlé et al., 2003). Paraffin-embedded tissues were cut to 3 μm thickness, mounted on SuperfrostPlus slides (Fisher Scientific, Pittsburgh, PA), deparaffinized and then rehydrated. Antigenicity lost during the fixation process was retrieved by heat treatment in a pressure cooker for 14 min as described (Brûlé et al., 2003). Non-specific binding sites were saturated by a 30 min incubation in blocking buffer: TBS (100 mM Tris pH 7.5, 150 mM NaCl), 1% bovine serum albumin and 1% skim milk. Tissue sections were incubated overnight at 4°C with a rabbit polyclonal antibody against human CAV1 (SC-894; Santa Cruz Biotechnology Inc.) diluted to 1:100 in blocking buffer. Negative control tissue sections were incubated similarly with or without normal rabbit serum. Primary antibody-CAV1 complexes were detected by incubation for 2 hr at room temperature with a monoclonal anti-rabbit IgG conjugated with alkaline phosphatase (Sigma Chemicals) diluted to 1:200 in blocking buffer. Tissue sections were washed three times in TBS, and incubated with the NBT/BCIP alkaline phosphatase substrate (Roche Applied Science, Laval, QC). Sections were mounted in 5% gelatin, 27% glycerol, and 0.1% sodium azide. Photographs were taken under bright field illumination using a Nikon Eclipse E800 microscope equipped with a digital camera (Nikon DXM 1200). Digital images were processed by Photoshop software (Adobe Systems Inc., San Jose, CA) and assembled by Illustrator software (Adobe Systems Inc.).

Statistical Analysis

Gene-specific signals (*CAV1*, *CAV2*) were normalized with corresponding *GAPD* signals for each sample. Homogeneity of variance between follicular groups and CL was verified by O'Brien and Brown–Forsythe tests. Corrected values of gene-specific mRNA levels were compared between follicular and CL groups by one-way ANOVA. When ANOVA indicated significant differences ($P < 0.05$), multiple comparisons of individual means for SF, DF, OF, and CL groups were compared by the Tukey–Kramer test ($P < 0.05$). Dunnett's test ($P < 0.05$) was used to compare individual means for the temporal hCG-induced follicular wall samples. Statistical analyses were performed using JMP software (SAS Institute, Inc., Carry, NC). Data were presented as means ± SEM.

RESULTS

Characterization of Bovine α-caveolin-1 cDNA

A bovine *CAV1* cDNA fragment (570 bp) was obtained from a previous gene expression profiling experiment using SSH screening that aimed at identifying differentially expressed genes in hCG-stimulated GC (Lévesque et al., 2002). This cDNA fragment was used as a probe to screen by hybridization a size-selected cDNA library of 2.3–2.7 kb generated from bovine GC that were collected 24 hr following injection of hCG. The full-length bovine *αCAV1* cDNA was cloned and consisted of 2,455 bp that included a 5′-untranslated region (UTR) of 62 bp, an open reading frame of 534 bp, and a 3′-UTR of 1859 bp containing four polyadenylation signals

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   1  AGTTCCCTTAAATCACAGCCCAGGGAAATCTACCCAGAGTCTTCAACCAGCCACGAGCC
  60 AGAATGTCGGGGGGCAAATACGTAGACTCAGAGGGACATCTCTACACTGTTCCCATCCGG
   1    MetSerGlyGlyLysTyrValAspSerGluGlyHisLeuTyrThrValProIleArg
 120 GAACAGGGCAACATCTACAAGCCCAACAACAAGGCTATGGCAGAGGAAATGAACGAGAAG
  20 GluGlnGlyAsnIleTyrLysProAsnAsnLysAlaMetAlaGluGluMetAsnGluLys
 180 CAAGTGTACGACGCGCACACCAAGGAGATAGATCTGGTCAACCGCGACCCCAAGCATCTC
  40 GlnValTyrAspAlaHisThrLysGluIleAspLeuValAsnArgAspProLysHisLeu
 240 AACGACGACGTGGTCAAGATTGATTTTGAAGATGTGATTGCAGAACCAGAAGGAACACAC
  60 AsnAspAspValValLysIleAspPheGluAspValIleAlaGluProGluGlyThrHis
 300 AGTTTCGATGGCATCTGGAAGGCCAGCTTCACCACCTTCACTGTGACAAAGTACTGGTTT
  80 SerPheAspGlyIleTrpLysAlaSerPheThrThrPheThrValThrLysTyrTrpPhe
 360 TACCGTTTGCTGTCTGCCCTCTTTGGCATCCCAATGGCACTCATCTGGGGCATTTACTTT
 100 TyrArgLeuLeuSerAlaLeuPheGlyIleProMetAlaLeuIleTrpGlyIleTyrPhe
 420 GCCATTCTCTCTTTCCTGCACATCTGGGCAGTTGTACCATGCATTAAGAGTTTCCTGATT
 120 AlaIleLeuSerPheLeuHisIleTrpAlaValValProCysIleLysSerPheLeuIle
 480 GAGATTCAGTGCATCAGCCGTGTCTATTCCATCTACGTCCACACCTTCTGTGACCCGCTG
 140 GluIleGlnCysIleSerArgValTyrSerIleTyrValHisThrPheCysAspProLeu
 540 TTTGAGGCTATTGGCAAAATATTCAGCAATATCCGCATCAACACGCAGAAAGAAATATAA
 160 PheGluAlaIleGlyLysIlePheSerAsnIleArgIleAsnThrGlnLysGluIle***
 600 ATGACATTTCAAGGATAGAAGTATATATGGTTCTTTCTTCCCTTTTAATTTTCTCAGTGC
 660 CGATTTCAAGTTGCTAGTATAGCAACAGCATATGAATGAATTTCCTGGTTCAGAACAAAG
 720 ATATCACTCTCTCAGTCTTCATAACTATTATTTTTCTCCTCTGAGCTATTGGGTTGTCTG
 780 TGTTGGGGGTTGTCTGAACTTTTAAAACCCATTTAAAATATTTTTCTTTCATTTGGATCA
 840 TTGTCCTATTGGCTGAGATATGAACCTATTGTTAAAAGATAACTTGAGAGAAATATGAAG
 900 AACTGAGGAGGAAAAAAAGAACTAACAACCTCAACTGCCTGTTCTAAAATGTCGATCATT
 960 TTATGGTAAGGGAAGTATTCAAGTATTCCAGGCTATTGTCACTGAGTGTACAGATATGTG
1020 GGCAGTTTTAAGCAAACTCTTCCCTCTGAAGTGTTTAGTGAATTGCTGCCATTCACTGTA
1080 GTGATTCAATGGGATCTAGTGATCTTCATCAAGTTAGAAAACATAATCTGCACATGATCT
1140 ATTGCCTTACTTTCTTGAAGTTTTAACCTGTGATAGATACTTGTATGCCTGGATATTTGT
1200 TACATAGATGATAACACCTAAGTGCCTTCCTGTTCTCCAGGTTTTCCTTTTAAAATAGGG
1260 TCCACCTCATCAACTTTCATTAGGTCAGCAGCCTTCCTGAAGACCAAAATTAGAAAAATC
1320 CATTACCTAGTTCTTCACACTTATTTCTGACTCCAAGATATGGGATCAGATGAAGTTCAT
1380 GTCTGTACTTGATCACACAACATCTTTATCCATATGGGGTATGGTCCACATCAGCCTCAT
1440 TAAATGAATTAAGGTGAATAAAGGGGCCAAGCCCTCTGGGCTGGCAGAAGTGGAAGCCA
1500 ACTTTCCCTGCCTCTCACTAGCTGAATGAGGTCAGCATGTCTATTCAGCTTCGTTTATTT
1560 TCAAGAATAATCACGCTTTCCTGACTCCAAACTAATCCATCACCGGGGTGGTTTAGTGGC
1620 TGAACATTGTGTTCCCTTTTCAGCTGATCAGTGGGCCTCCGGGGAAGGGCTCATAAAATG
1680 GAGGCCATTGTGTGAGACTGTCAGAGTTGTTGCAAATGTGACCCCTTCAAATTAAAGCAC
1740 TTGCAACTGCCTGTTATGCTGTGACACATGGCCCCTCCCCCTGCCACGAGCTTTGGACTT
1800 AAGCCAAGCGTCACTTTGCTCAGAGAGAAGATGGGGGAGGAGGCAGTAATAAAAGATGGA
1860 GGTAATTTTGCTGGAATAAATTCAAATTCTTCTGAATTCAAACTGAGGAATTTTACCTGT
1920 AAACCTGAGTCATACAGAAAGCTGCCTGGTACATCCAAAAGCTTTTTATTCCTCCTGCTC
1980 ATAGTAAGATTCTTCCCTGGGGGACTTTATTTTTAACCTTCAGTTATGCTTTTATTTCCA
2040 TACACCTGTTAGAATTCTGCTTGGTTTCTTTTTTTGCCTCTTCCAGTTTTCCTGGCACTT
2100 TTAATCGCCAACCTGTTACCTATTTAGGTTTTCTGCATTAAAACAGACACTGGCATGGAC
2160 ATAGTTTTACTTTTAAACTGTGTACATAACTGAAAATGTACTATACTGAATAATTTTTAA
2220 GTGAAAAGTTTTTTTATCTTTATATGAGGAAAATCACTTGGGAAATTGTTTTGTGATTCA
2280 ATCTGTAAACTGTGTATCCCAAGACATGTCTGTTCTATATGGATGCTTATTCCCTTGTGC
2340 AAATCAAGTGCTGGTCCAAAAGACTGCTGAAATTTTTATATGCTTACTGATATTTTACAC
2400 TTTTTTTATCTTGCATGTCCTGTAATAAGTCTACACAATAAAAATGTTTAACAGTC
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Fig. 1. Nucleotide and amino acid sequences of bovine αCAV1. The bovine *αCAV1* cDNA was cloned by library screening, as described under Materials and Methods, and amino acids deduced from cDNA. Nucleotides and amino acids are numbered at the left; amino acid numbering begins at the first methionine. In the 3′-UTR region, *asterisks* represent the stop codon, and the four polyadenylation signals are underlined. The poly(A)$^+$ tail is not represented. The nucleotide sequence was submitted to Genbank (accession number: AY823915).

(AATAAA) followed by a poly(A)$^+$ tail (Fig. 1). The coding region of bovine *αCAV1* cDNA encodes a 178-amino acid protein, with a theoretical molecular mass (Mr) of 20,593 and an isoelectric point (pI) of 5.6. Amino acid homology search in GenBank by PsiBlast revealed orthologous proteins with an overall identity level of 100% to ovine (NP_001009477), 99% to porcine (AAR16300), 97% to rat (AAR16308), and 96% to human (AAP36880) and mouse (AAR16290) proteins. When compared to these species, protein domains of importance are conserved in bovine αCAV1 (Fig. 1), including: a caveolin-1 consensus motif (Phe^{68}–Pro^{75}), a caveolin-scaffolding domain (CSD; Asp^{82}–Arg^{101}), a potential hairpin loop structure that inserts into the membrane (Ala^{105}–Leu^{125}), a potential phosphorylation site (Tyr^{14}), and potential S-palmitoylated cysteines (Cys^{133}, Cys^{143}, Cys^{156}) located at the carboxyl-terminus.

Expression of αCAV1 and CAV2 mRNAs in Granulosa Cells and CL

The expression profile of *αCAV1* mRNA was studied first by virtual Northern analysis using GC collected from follicles obtained at different developmental stages: SF (2–4 mm), DF at day 5 of the estrous cycle, OF 24 hr following injection of an ovulatory dose of hCG, and corpus luteum (CL) at day 5. A single transcript estimated at 2.7 kb for *αCAV1* was observed and was shown to be expressed predominantly in GC of hCG-induced OF (data not shown). Semiquantitative RT-PCR demonstrated a 6.5-fold increase in *αCAV1* mRNA in GC of hCG-stimulated OF compared to DF ($P < 0.0001$; Fig. 2); *αCAV1* mRNA was also detected in CL albeit at a low level. In a parallel analysis, *CAV2* mRNA showed a similar pattern of expression as *CAV1* with a 1.7-fold increase in GC of OF compared to DF ($P < 0.0007$; Fig. 2) whereas the transcript level was lower in GC of SF. These results underscore that *αCAV1* and *CAV2* mRNA levels are regulated in GC in association with the stage of follicular development, however, the level of induction by the LH/hCG surge in GC of OF is more intense for *αCAV1* than *CAV2*.

Regulation of αCAV1 and CAV2 mRNAs in Follicles During the Periovulatory Period

Since hCG induced *αCAV1* mRNA in GC of OF 24 hr following its injection, semiquantitative RT-PCR was used to study the regulation of *αCAV1* and *CAV2* mRNAs in follicular walls of OF isolated at different times between 0 and 24 hr post-hCG treatment. The RT-PCR analysis of *αCAV1* mRNA showed a significant upregulation of the transcript level after hCG treatment, with an initial increase of 5.2-fold at 12 hr that reached a maximum of 8.5-fold at 24 hr when compared to mRNA level preceeding hCG injection at 0 hr ($P < 0.0018$; Fig. 3). This result was confirmed by virtual Northern blot analysis (data not shown). In contrast, *CAV2* transcript level did not vary significantly in follicles collected at various times during the periovulatory period ($P < 0.16$; Fig. 3). Quality of follicular wall samples during the periovulatory period was validated

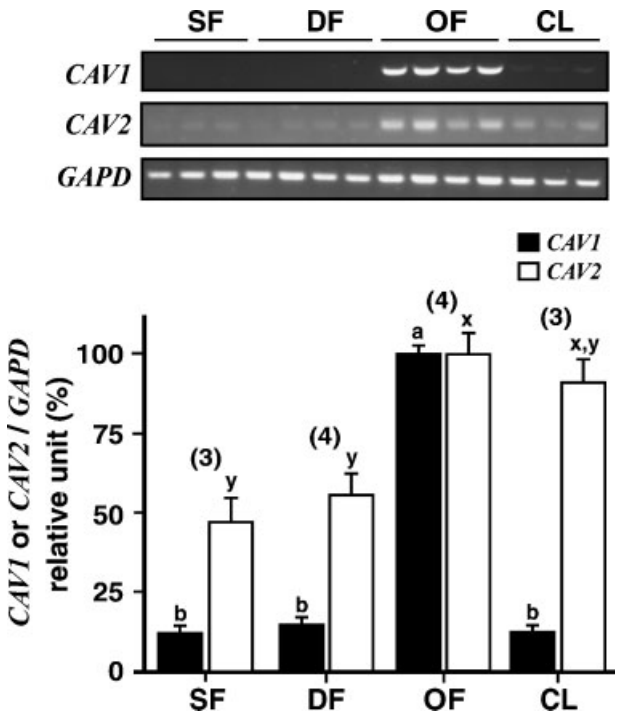

Fig. 2. Expression of *αCAV1* and *CAV2* transcripts in bovine granulosa cells and CL. Total RNA was extracted from bovine granulosa cells collected from 2 to 4 mm small follicles (SF), dominant follicles (DF) at day 5 of the estrous cycle, ovulatory follicles (OF) 24 hr after injection of hCG (OF) and corpora lutea (CL) from day 5 of the estrous cycle, then served for mRNA expression analyses using semiquantitative RT-PCR as described under Materials and Methods. The control gene *GAPD* (710 bp) showed no significant difference in mRNA expression levels between groups. The *αCAV1* (437 bp) and *CAV2* (960 bp) PCR fragments were normalized with their corresponding *GAPD* signal, and the results are presented as a relative change in ratio between groups. Different letters denote samples that differed significantly ($P < 0.05$). Data are presented as means ± SEM, and the number of independent samples (i.e., animals per group) is indicated in parenthesis.

by RT-PCR analysis of control genes including cytochrome P450 family 19 subfamily 1 (*CYP19A1*), a gene known to be down-regulated by LH/hCG, and prostaglandin-endoperoxide synthase 2 (*PTGS2*), a gene known to be up-regulated by LH/hCG (Diouf et al., 2006).

Regulation and Localization of αCAV1 Protein in Ovulatory Follicles

To determine whether the increase in *αCAV1* mRNA in bovine OF after hCG treatment was associated with changes in protein level, the regulation of αCAV1 was studied in GC protein extracts isolated from follicles collected between 0 and 24 hr post-hCG treatment. Immunoblot analysis showed that the antibody recognized a Mr 22,000 band that was weakly expressed in GC preceeding hCG treatment. The increase in protein level was detected initially at 12 hr post-hCG and reached a maximum at 24 hr (Fig. 4A). The expression of GSTA1, used as a control protein, showed no significant difference between samples (Fig. 4B). Cellular localization of αCAV1 protein expression in follicles was

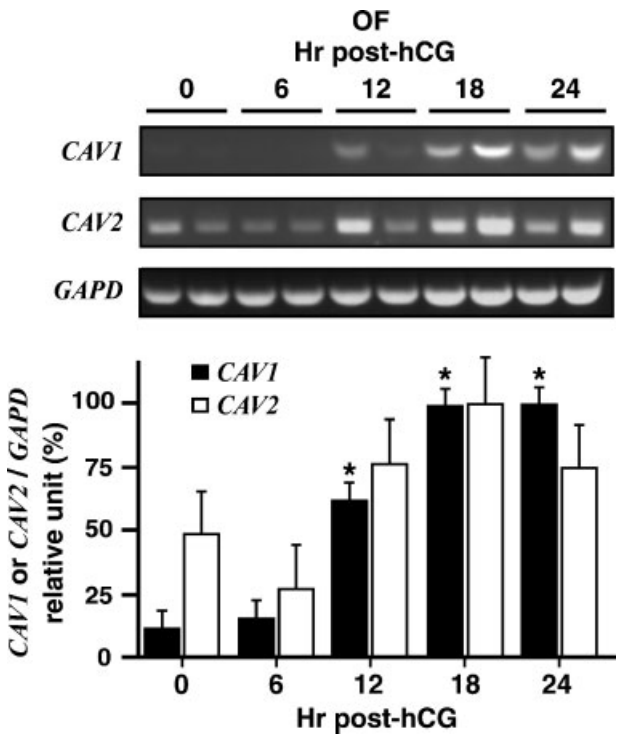

Fig. 3. Regulation of *αCAV1* and *CAV2* transcripts by hCG in bovine follicles during the periovulatory period. Total RNA was extracted from preparations of bovine follicular wall obtained from ovulatory follicles isolated between 0 and 24 hr after injection of hCG then used in mRNA expression analyses by semiquantitative RT-PCR as described under Materials and Methods. The control gene *GAPD* (710 bp) showed no significant differences in mRNA expression levels between groups. The *αCAV1* (437 bp) and *CAV2* (960 bp) PCR signals were normalized with their corresponding *GAPD* signals, and the results are presented as a relative change in ratio between groups. Bars marked with an asterisk are significantly different ($P < 0.05$) from 0 hr post-hCG. Data are presented as means ± SEM, representing two independent animals per time point.

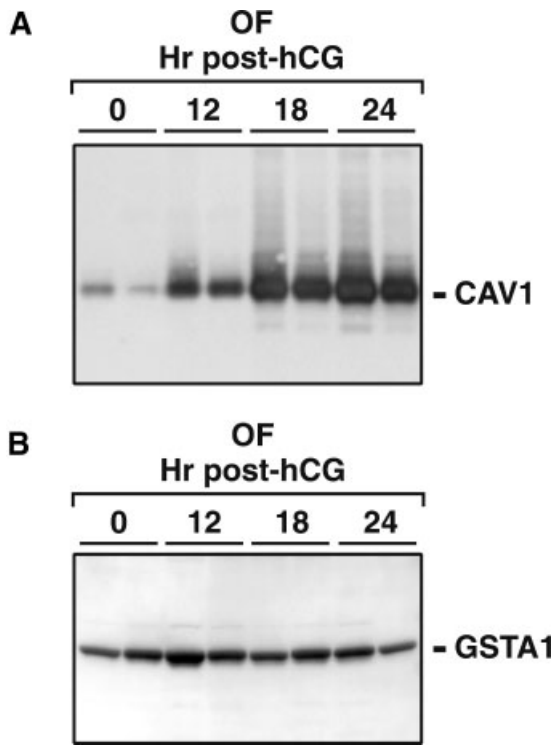

Fig. 4. Regulation of αCAV1 protein by hCG in bovine follicles. Granulosa cells were isolated from bovine ovulatory follicles between 0 and 24 hr after hCG treatment. Protein extracts (50 μg) were analyzed on a one-dimensional 18% SDS–PAGE and immunoblotted as described under Materials and Methods. Immunoblotting was performed using (**A**) a polyclonal antibody against human CAV1 (1:500) revealing a Mr 22,000 protein band or (**B**) a polyclonal antibody against bovine GSTA1 (1:3,000) revaling a Mr 28,000 protein band. Two distinct follicles from independent animals per time point are represented.

determined by immunohistochemistry. Strong immunostaining for αCAV1 was detected in endothelial cells of all antral follicles and CL (Fig. 5A). No immunoreactive signal was detected in GC of dominant or preovulatory follicles collected at day 5 or 7 of the estrous cycle (Fig. 5A, B) and small antral follicles (2–4 mm; Fig. 5E). At 18 and 24 hr after hCG injection, αCAV1 protein was clearly induced in GC that presented heterogeneity in the labeling pattern (Fig. 5C, D). GC of small antral follicles adjacent to the ovulatory follicle were not stained (Fig. 5E). Theca and luteal cells did not stain for αCAV1 (Fig. 5A–E).

DISCUSSION

This study demonstrates that αCAV1 expression at the mRNA and protein levels is acutely increased in GC of bovine OF, whereas *CAV2* expression was stimulated by hCG albeit to a moderate degree when compared to *αCAV1*. Caveolins are known to act as scaffolding proteins and are protein markers of caveolae (Williams and Lisanti, 2004). Caveolae are 50–100 nm vesicular invaginations of the plasma membrane where caveolins are found predominantly but they are also present in Golgi, endoplasmic reticulum (ER), vesicles, and in cytosolic locations such as ER lumen and secretory vesicles in a wide variety of tissue and cell types (Mora et al., 1999; Ostermeyer et al., 2001; Cohen et al., 2004; Williams and Lisanti, 2004). Three members of the caveolin gene family have been identified: caveolin 1 (*CAV1*), caveolin 2 (*CAV2*), and caveolin 3 (*CAV3*). These caveolin genes and proteins share significant homology, and proteins are highly conserved throughout evolution (Cohen et al., 2004). CAV1, also termed vesicular integral-membrane protein of 21 kDa (VIP21), and CAV3 proteins are both independently necessary and sufficient to drive caveolae formation whereas CAV2 requires the presence of CAV1 for proper membrane targeting and stabilization (Cohen et al., 2004). The functional roles attributed to CAV1 are diverse and range from vesicular transport such as endocytosis and transcytosis (Nabi and Le, 2003; Pelkmans et al., 2004; Quest et al., 2004), to cholesterol homeostasis (Murata et al., 1995; Graf et al., 1999; Schlegel et al., 2000), regulation of signal transduction and suppression of cellular transformation (Okamoto et al., 1998; Liu et al.,

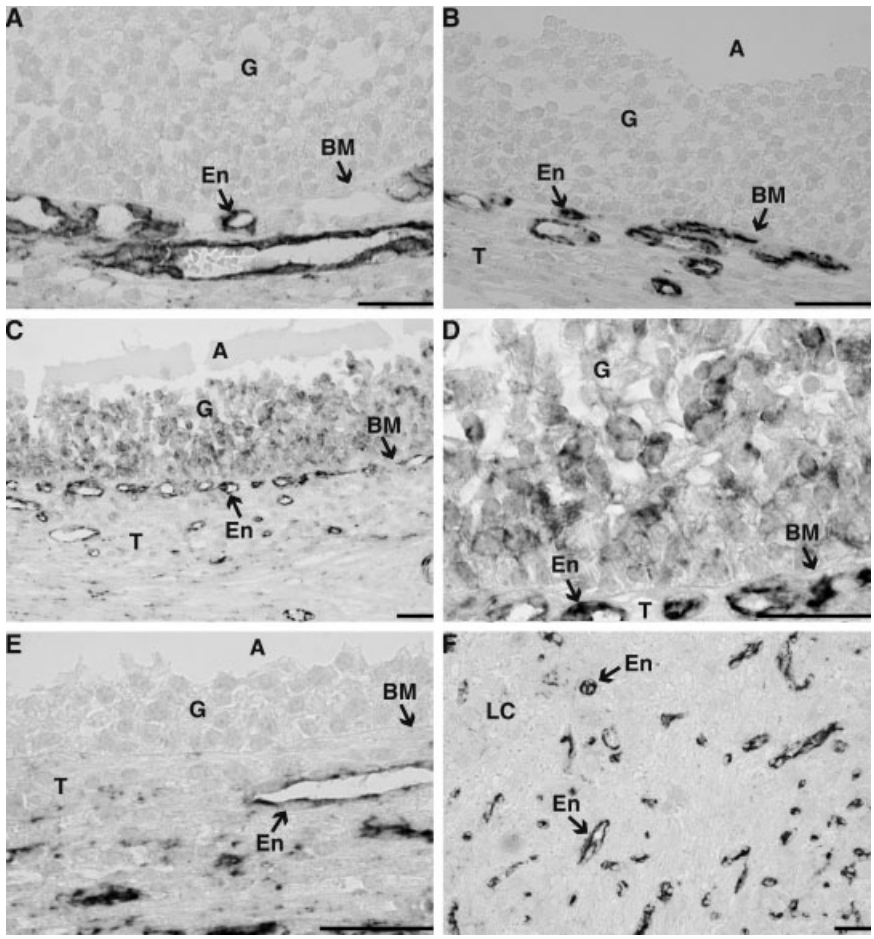

Fig. 5. Immunohistochemical localization of αCAV1 in bovine ovulatory follicles and CL. Immunohistochemistry was performed on formalin-fixed sections of ovulatory follicles isolated preceeding and following hCG injection, as described under Materials and Methods. The scale bar is equal to 0.05 mm in all figures. Staining was absent when the primary antibody was omitted (data not shown). **A**: DF at day 5 of the estrous cycle; (**B**) preovulatory follicle at day 7; (**C**) ovulatory follicle 24 hr post-hCG; (**D**) enlargement of OF presented in C; (**E**) small antral follicle (2 mm) adjacent to the ovulatory follicle at 24 hr post-hCG; (**F**) CL at day 5 of the estrous cycle. (A, antrum; BM, basal membrane; En, endothelial cells; G, granulosa; LC, luteal cells; T, theca.)

2002; Razani et al., 2002; Cohen et al., 2004; Williams and Lisanti, 2005).

Two isoforms of CAV1 have been identified in other species: the αCAV1 consists of 178 amino acids whereas the βCAV1 lacks the first 31 amino acids of the αCAV1 (Scherer et al., 1995; Fujimoto et al., 2000; Razani et al., 2002). The α and βCAV1 derived from *CAV1* gene are generated from distinct mRNAs that are regulated independently at the transcriptional level by alternative promoters (Kogo et al., 2004). In the present study, RT-PCR analysis of *CAV1* mRNA using oligos spanning amino acid G^4–Q^{175} of bovine αCAV1 revealed a single amplicon corresponding to the α-isoform. Furthermore, a single protein band of 22 kDa was observed in immunoblotting analysis of bovine GC, which corresponds to αCAV1, confirming the RT-PCR observations. Characterization of *αCAV1* mRNA revealed four polyadenylation signals in its 3′-UTR segment. The precise biological reason of these multiple adenylation signals in αCAV1 are unknown, but as suggested for other genes, they may increase the stability of *αCAV1* mRNA or the efficacy of translation through tissue-specific polyadenylation factors (De Moor et al., 2005). As observed for orthologous proteins, the bovine CAV1 protein contains a conserved hydrophobic membrane spanning domain (A^{105}–L^{125}), which adopts a hairpin structure within membrane lipids resulting in amino- and carboxy-termini located in the cytoplasm (Razani et al., 2002). The three cysteines at the C-terminus (C^{133}, C^{143}, C^{156}) are conserved and may be palmitoylated thus helping to stabilize the protein at the membrane (Razani et al., 2002; Cenedella et al., 2006). The CAV1 scaffolding domain (D^{82}–R^{101}) allows interactions with diverse proteins by recognizing a specific caveolin binding motifs (AXAXXXXA and AXXXXAXXA; where A is an aromatic amino acid) localized in intracellular signaling proteins (Couet et al., 1997). The protein–protein interaction capacity enables the formation of high molecular weight homo- and hetero-oligomers. CAV1 may also be phosphorylated on Y^{14} in response to a number of growth factors that regulate its interaction with signal transducing proteins (Cao et al., 2002; Razani et al., 2002; Labrecque et al., 2004; Repetto et al., 2005).

Expression of CAV1 was induced in bovine GC following injection of an ovulatory dose of hCG, but its role at the time of ovulation remains hypothetical. A role for CAV1 in cholesterol transport in GC remains elusive since dominant or preovulatory follicles known to synthesize high concentration of estradiol do not express CAV1 (Voss and Fortune, 1993a,b; Bédard et al., 2003; Fayad et al., 2004; Ndiaye et al., 2005). Furthermore, at the time of ovulation, steroid hormone synthesis decreases in GC when CAV1 expression increases (Voss and Fortune, 1993a,b; Ndiaye et al., 2005), and despite the high progesterone synthesis by luteal cells (Niswender, 2002), CAV1 expression in bovine luteal cells was not detected by immunohistochemistry. Interestingly, CAV1 was shown to interact with phospholipase A2 group IVA (PLA2G4A also known as cPLA2α) (Gaudreault et al., 2004) and prostaglandin-endoperoxide synthase 2 (PTGS2 also known as COX2) (Liou et al., 2001), two enzymes involved in prostaglandin synthesis. PLA2G4A releases arachidonic acid from membrane phospholipids, which is then converted to PGH2 by PTGS2 and then further to PGE by PGE synthase (Filion et al., 2001). PLA2G4A and PTGS2 are induced by the LH/hCG preovulatory surge in bovine GC (Ndiaye et al., 2005; Diouf et al., 2006). CAV1 was shown to inhibit PLA2G4A enzymatic activity (Gaudreault et al., 2004) and to co-localize with PTGS2 (Liou et al., 2001). Collectively, these results suggest that CAV1, through its compartmentalization and interaction with PLA2G4A and PTGS2, could modulate prostaglandin production during ovulation.

Since the preovulatory LH/hCG surge induced CAV1 expression in GC, a role for CAV1 in modulating LHCGR signaling is possible. It is known that upon ligand activation, LHCGR cluster at the cytoplasmic membrane (Hunzicker-Dunn et al., 2003), are phosphorylated and complexed with β-arrestin 2, and are internalized via clathrin-coated pits (Marchese et al., 2003; Lefkowitz and Shenoy, 2005). These complexes are then targeted to lysosomes for degradation (Bhaskaran and Ascoli, 2005). However, several G-protein coupled receptors such as endothelin, bradykinin, muscarinic acetylcholine, and β-adrenergic receptors have been localized to caveolae, and CAV1 is known to directly interact with the α-subunit of G-proteins (Razani et al., 2002). It is thus plausible that an increase in CAV1 following the LH/hCG surge may inhibit LHCGR signaling through clathrin-independent endocytosis (Nichols and Lippincott-Schwartz, 2001). The recent description of CAV1-null mice further suggest, that CAV1 may act as a tumor suppressor and regulator of cell proliferation by modulating the signaling of various growth factors (Williams and Lisanti, 2005). These observations suggest that CAV1, at the time of ovulation, may be involved in regulating growth factor signaling, thereby modulating GC division.

The expression of both *CAV1* and *CAV2* mRNA was detected in GC but the expression of *CAV1* is acutely regulated in comparison to *CAV2*. Expression of *CAV2* mRNA was detected in GC of SF and DF, and the increase following hCG injection was less pronounced when compared to *CAV1*. CAV2 is unable to form caveolae by itself and requires interaction with CAV1 to be incorporated into caveolae (Williams and Lisanti, 2004). The formation of caveolae is thus driven by the presence of CAV1. The presence of CAV1 protein in GC extracts of small antral follicles was detected by immunoblotting and may have resulted from minor contamination of endothelial cells when GC were mechanically dislodged from follicular walls. Similarly, the detection of basal levels of *CAV1* mRNA in follicular wall samples at time 0 hr post-hCG likely reflects the endothelial cell contribution of CAV1 *mRNA* to these samples. This explanation is supported by the immunohistochemical observations, which clearly demonstrate the high expression of CAV1 in endothelial cells of capillaries adjacent to the basal membrane of the granulosa layer, and the absence of CAV1 in GC of SF and DF. The high expression of CAV1 in endothelial cells is well described in the literature (Razani et al., 2002), and may serve in transcytosis of blood-born nutrients reaching the GC layer. The expression of CAV1 mRNA and proteins in CL was associated with endothelial cells of capillaries since luteal cells were not immunolabeled for CAV1.

In summary, this report demonstrates for the first time an LH/hCG-dependent induction of *CAV1* mRNA and protein in GC in vivo. Since CAV1 can associate through its scaffolding domain to many proteins thus inhibiting their biological action, the upregulation of CAV1 expression is likely to be important for controlling the increase in membrane signaling occurring at the time of ovulation and luteinization.

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