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Expression of Phospholipase A2 Group IVA (PLA2G4A) Is Upregulated by Human Chorionic Gonadotropin in Bovine Granulosa Cells of Ovulatory Follicles[1]

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ABSTRACT

Prostaglandins are required for the ovulatory process, and their biosynthesis depends on the initial release of arachidonic acid from membrane phospholipids. We hypothesized that phospholipase A2 group IVA (PLA2G4A) expression is upregulated in granulosa cells (GC) at ovulation. We have characterized bovine *PLA2G4A* cDNA, and investigated its spatiotemporal regulation at the mRNA and protein levels in hCG-induced ovulatory follicles and in vitro, using forskolin-stimulated GC. Regulation of *PLA2G4A* mRNA expression was studied in GC obtained from bovine follicles collected at different developmental stages: small follicles (2–4 mm), dominant follicles at Day 5 (D5) of the estrous cycle, ovulatory follicles 24 h following injection of hCG, and corpus luteum at D5. *PLA2G4A* mRNA increased by 14-fold in GC of hCG-stimulated versus dominant follicles ($P < 0.0001$). Follicular walls obtained from ovulatory follicles recovered at 0, 6, 12, 18, and 24 h post-hCG injection showed an initial 16-fold increase in *PLA2G4A* transcript at 12 h that reached a 45-fold increase at 24 h, as compared to 0 h ($P < 0.0001$). Immunoblots of GC extracts showed an initial induction of the PLA2G4A protein at 18 h post-hCG, reaching a maximum at 24 h. Immunohistochemistry observations showed that PLA2G4A signal was mainly observed in mural GC compared to antral GC in hCG-stimulated follicles. Stimulation of cultured bovine GC with 10 μM of forskolin caused an increase in *PLA2G4A* mRNA and protein. Ovulation is associated with an LH/hCG-dependent induction of PLA2G4A in GC via the adenylyl cyclase/cAMP pathway.

cPLA2α, follicle, follicular development, gene expression, granulosa cells, ovary, ovulation, phospholipase A2, PLA2A, PLA2G1B, PLA2G4A

INTRODUCTION

In mammals, the ovulatory process begins at the time when the endogenous 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. This gonadotropin-induced process is controlled by temporal and spatial expression of specific genes, and presents features reminiscent of an acute inflammatory reaction [1, 2]. Modern methods in molecular biology, such as mRNA differential display, cDNA array, and suppression subtractive hybridization (SSH) allow the detection of differentially expressed genes during the process of follicular growth and ovulation [2–4]. Transcription of genes in granulosa cells (GC) that controls the growth of a bovine dominant or preovulatory follicle is rapidly downregulated as a consequence of LH-mediated increases in intracellular signaling [5, 6]. In conjunction with the termination of specific gene expression in preovulatory follicles, LH/hCG induces expression of genes involved in ovulation and luteinization, as shown in rodents [2]. For ovulation, many genes were found to be induced or upregulated by the LH surge using rodent models, such as prostaglandin-endoperoxide synthase 2 (PTGS2) [7], progesterone receptor [8], CCAAT/enhancer-binding protein beta [9, 10], and early growth response protein-1 [11]. These were shown to be obligatory for ovulation to occur because the process is impaired in mice null for these genes. Most data on the temporal pattern of gene expression arising during ovulation have been obtained using whole ovarian RNA extracts from the immature eCG- and hCG-stimulated rodent ovaries, which may conceal identification of genes specifically induced in GC. Using GC isolated from hCG-induced ovulatory follicles from bovine adult females, SSH was applied to identify upregulated expression of specific genes that occurs during ovulation [12]. The latter study identified a cDNA fragment that corresponded to the 3′-untranslated region (3′-UTR) of the bovine phospholipase A2 group IVA (*PLA2G4A*), also known as the cytosolic PLA2 alpha (*cPLA2α*). Thus, *PLA2G4A* was identified as a potential candidate gene induced in GC following stimulation by the LH/hCG preovulatory surge.

The PLA2 family is subdivided on the basis of size, secretory capabilities, and calcium requirements into three subfamilies: the secreted low-molecular-weight Ca^{2+}-dependent enzymes (sPLA2-IB, sPLA2-IIA), the cytosolic high-molecular-weight Ca^{2+}-dependent enzymes (cPLA2s), and the Ca^{2+}-independent isoforms (iPLA2) [13–15]. The cPLA2 subfamily is composed of three enzymes that share some structural features without being functionally redundant: PLA2G4A (cPLA2α), PLA2G4B (cPLA2β) and PLA2G4C (cPLA2γ) [16, 17]. Most of the PLA2s are widely expressed in different tissues, where they are involved in different biological processes such as inflammation, tissue repair, and apoptosis [18, 19]. The biological function of PLA2 enzymes is to catalyze hydrolysis of the sn-2 position of membrane glycerophospholipids, leading to production of free fatty acids

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and lysophospholipids, two precursors of potent bioactive mediators. This reaction is of particular importance if the esterified fatty acid is arachidonic acid (AA). Thus, the first regulated step involved in prostaglandin formation is the release of AA from membrane glycerophospholipids, which is selectively done by cPLA2s [20]. Conversion of AA is further processed by downstream metabolic enzymes like cyclooxygenases to eicosanoids, including prostaglandins (PGs) and leukotrienes [17], known to impact ovulatory mechanisms.

In relation to follicular growth and ovulation, increase in ovarian PLA2 activity in response to LH/hCG injection was first reported in rodents using whole protein ovarian extract [21]. Because a fragment of *PLA2G4A* cDNA was identified as upregulated during ovulation in the bovine species [12], we hypothesized that expression of the *PLA2G4A* gene may be induced by the LH/hCG preovulatory surge in GC, thereby contributing to the increase in AA substrate required for the synthesis of PGs involved in the ovulatory process. In the present study, the bovine ovulatory follicle was used as the in vivo model to investigate the regulation of *PLA2G4A* during hCG-induced ovulation/luteinization. The regulation of *PLA2-G4A* was then further studied in vitro by analyzing the stimulation of forskolin (FSK) on *PLA2G4A* expression using an established bovine GC culture system. The specific objectives of this study were to clone bovine *PLA2G4A* cDNA and to determine the spatiotemporal expression profile of *PLA2G4A* mRNA and protein in ovulatory follicles following hCG treatment and in FSK-stimulated GC.

MATERIALS AND METHODS

Cloning of Bovine PLA2G4A cDNA

Isolation of the full-length bovine *PLA2G4A* cDNA was performed by screening a size-selected cDNA library. Initially, the size of the full-length bovine *PLA2G4A* cDNA was estimated by performing a virtual Northern blot analysis. Briefly, total RNA was isolated from GC that were obtained from bovine ovulatory follicles 24 h following an hCG injection [6], and transformed into cDNA by the SMART cDNA synthesis method (BD Biosciences Clontech) as previously described [12]. The cDNAs were separated by gel electrophoresis, transferred onto a nylon membrane, and hybridized with a bovine *PLA2G4A* radioactive probe (760 bp) generated from a previous SSH screening experiment [12]. Once the size of the full-length bovine *PLA2G4A* cDNA was determined, total SMART cDNAs from hCG-stimulated GC were size-fractionated by agarose gel electrophoresis, and cDNAs from 2.5 to 3.5 kb were purified and used to construct a size-selected cDNA library based on the pDrive plasmid (Qiagen PCR cloning kit; Qiagen) that was then screened by radioactive hybridization as previously described [12]. Positive *PLA2G4A*-hybridizing bacterial colonies were grown, their plasmid contents were isolated (QIA-prep, Qiagen), and the size of the cloned cDNA was analyzed following an EcoR1 digestion and gel electrophoresis analysis. The cDNAs were sequenced via the dideoxy sequencing method (Big Dye Terminator 3.0; ABI Prism, Applied BioSystems, PE) that were analyzed on an ABI Prism 310 sequencer (Applied Biosystems). Nucleic acid sequences were analyzed by BLAST 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 cyclic crossbred heifers that were held at the large animal complex of the Faculty of Veterinary Medicine, as previously described [6, 22]. Briefly, estrous cycle was synchronized with $PGF_{2\alpha}$ (25 mg, i.m.; Lutalyse, Upjohn) and ovarian follicular development was monitored by daily transrectal ultrasonography until ovariectomy. Dominant follicles (DF; n = 4 cows) were defined as >8 mm and growing, whereas subordinate follicles were either static or regressing. They were obtained on the morning of Day 5 of the estrous cycle (Day 0 = day of estrus) by ovariectomy (via colpotomy). Ovulatory follicles (OF) were obtained following an injection of 25 mg of $PGF_{2\alpha}$ on Day 7 of the synchronized estrus 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, i.v.; APL, Ayerst Lab) was injected 36 h after the induction of luteolysis, and the ovary bearing the hCG-induced OF was collected by ovariectomy at 0, 6, 12, 18, and 24 h after hCG injection (n = 2–4 cows/time point). Follicles were dissected into preparations of follicular wall (theca interna with attached GC) [22] or further dissected into separate isolates of GC [6]. Ovariectomies were also performed on Day 5 of the synchronized estrous cycle to obtain CL (n = 3 cows). Additionally, GC were collected from 2–4 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 previously described [23]. The concentration of total RNA was quantified by measuring 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.

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) to generate the first strand cDNA using the SMART PCR cDNA synthesis kit (user manual PT3041–1; BD Biosciences Clontech) [6]. Second cDNA strands were produced with the SMART II 5′-anchored oligo, and PCR-amplified for 18 cycles using Advantage 2 DNA polymerase (BD Biosciences Clontech). To perform semiquantitative RT-PCR, SMART cDNA pools were diluted 10-fold in H_2O, and 1–2 μl was 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 cDNA sequence for *PLA2G4A* (sense, 5′-CTTGCATTCTACACGTGATGTGCC-3′; antisense, 5′-GATGTATTGAGATTCAAGCCCAGC-3′; GenBank AY363688), phospholipase A2 group IB (*PLA2G1B*, also known as pancreatic PLA2A; sense, 5′-GAGACTCCTGGTGTTGGCTGCTC-3′; antisense, 5′-TTCTTGTCCAGGTTCTTGTGCTCC-3′; GenBank NM_174646), cytochrome P450 family 19 subfamily 1 (*CYP19A1*; sense, 5′-GTCCGAAGTTGTGCCTATTGCCAGC-3′; antisense, 5′-CCTCCAGCCTGTCCAGATGCTTGG-3′; GenBank NM_174305), prostaglandin-endoperoxide synthase 2 (*PTGS2*; sense, 5′-GCATTCTTTGCCCAGCACTTCACCC-3′; antisense, 5′-CTATCAGGATTAGCCTGCTTGTCTGG-3′; GenBank AF031698), and glyceraldehyde-3-phosphate dehydrogenase (*GAPD*; sense, 5′-TGTTCCAGTATGATTCCACCCACG-3′; antisense, 5′-CTGTTGAAGTCGCAGGAGACAACC-3′) [6]. 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 (14–20 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, and 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).

Cell Extracts and Immunoblot Analysis

Tissues and cells were homogenized in M-PER buffer (Pierce) that was supplemented with a mix of protease inhibitors (Complete; Roche Applied Science) as recommended by the manufacturer's protocol. Complete lysis of GC was achieved with multiple passages through a 25-gauge needle attached to a 3-ml syringe. CL were homogenized with a polytron PT1300D (7000 rpm; Kinematica AG). 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 until electrophoretic analyses were performed. Protein concentration was determined by the Bradford method (Bio-Rad Protein assay) [24]. Protein extracts (100 μg proteins/sample) were heat-treated (5 min, 100°C), size-fractionated via one-dimensional SDS-PAGE, and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham Pharmacia Biotech). Immunoblots were performed as previously described [25]. Membranes were incubated with the monoclonal antibody against human PLA2G4A (1:100; SC-454; Santa Cruz Biotechnology Inc.) and detected with a horseradish peroxidase-linked sheep anti-mouse secondary antibody (1:20 000 dilution; Amersham Pharmacia Biotech). To analyse PTGS2 protein, membranes were incubated with the rabbit anti-human PTGS2 antibody (1:5,000; MF243) [26] and detected with a horseradish peroxidase-linked donkey 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 protein [27]. Membranes were incubated with a rabbit anti-bovine GSTA1 antibody (1:3000 dilution), detected with an alkaline phosphatase-linked goat anti-rabbit secondary antibody (1:20 000 dilution;

Sigma Chemicals) and revealed using NBT/BCIP substrate (Roche Molecular Biochemicals). Immunoblots were digitized using a ScanMaker 9800XL flatbed scanner (Microtek Lab, Inc.).

Primary Granulosa Cell Cultures

Primary cultures of bovine GC were performed as previously described [28]. Briefly, cells were seeded in cultures at a density of 2×10^6/100 mm plates in minimal essential medium supplemented with L-glutamine, nonessential amino acids, 2% fetal bovine serum, insulin (1 μg/ml), transferin (5 μg/ml), and penicillin (100 units/ml)-streptomycin (100 μg/ml), and incubated at 37°C in a humidified atmosphere of 5% CO_2. Prior to addition of FSK, cells were starved overnight in fresh culture media in the absence of serum. After incubation for various times in the presence of FSK (10 μM), cells were harvested and submitted to total RNA and protein extractions. Extraction of total RNA from GC was performed using TRIzol reagent (Invitrogen Life Technologies). RNA samples (100 ng) served for the RT-PCR analysis using the One-Step RT-PCR System in accordance with the manufacturer's instructions (Qiagen), with sense and antisense primers specific for *PLA2G4A* and *GAPD* as described above. The reaction conditions were one cycle at 48°C for 30 min and 95°C for 15 min, followed by 35 PCR cycles of 94°C for 30 sec, 59°C for 1 min, and 72°C for 2 min. Following PCR amplification, reaction products were electrophoresed on 1% TAE-agarose gels. Whole cell protein extracts were isolated as described previously, and protein concentrations were determined as described above. Samples (50 μg of protein/well) were resolved by one-dimensional SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were immunoblotted and revealed as described above.

Immunohistochemical Localization of PLA2G4A

Immunohistochemical staining was performed on PBS-buffered formalin-fixed tissues as previously described [25]. Paraffin-embedded tissues were cut to 3-μm thickness, mounted on SuperfrostPlus slides (Fisher Scientific), deparaffinized and then rehydrated. Antigenicity lost during the fixation process was retrieved by heat treatment in a pressure cooker for 14 min as previously described [25]. Nonspecific binding sites were saturated by a 30-min incubation in blocking buffer: TBS (100 mM Tris pH 7.5, 150 mM NaCl), 1% BSA, and 1% fat-free skim milk. Tissue sections were incubated overnight at 4°C with a monoclonal antibody against human PLA2G4A (SC-454; Santa Cruz Biotechnology Inc.) diluted to 1:100 in blocking buffer. Negative control tissue sections were incubated similarly with or without normal mouse serum. After three 5-min washes in TBS, primary antibody-PLA2G4A complexes were detected by incubation for 2 h at room temperature with a rabbit anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich) diluted to 1:40 in blocking buffer. Tissue sections were washed three times in TBS and incubated with the NBT/BCIP alkaline phosphatase substrate (Roche Applied Science). Sections were mounted in 5% gelatin, 27% glycerol, and 0.1% sodium azide. Photographs were taken under brightfield 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.) and assembled by Illustrator software (Adobe Systems Inc.).

Statistical Analysis

Gene-specific signals 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 by one-way ANOVA in GC obtained from bovine follicles collected at different developmental stages or CL, and in follicular wall preparations following induction by hCG. When ANOVA indicated significant differences ($P < 0.05$), the Tukey-Kramer test was used for multiple comparisons of individual means. Data were presented as means ± SEM. Statistical analyses were performed using JMP software (SAS Institute, Inc.).

RESULTS

Characterization of Bovine PLA2G4A cDNA

A bovine *PLA2G4A* cDNA fragment (760 bp) was initially obtained from a previous gene expression profiling experiment using an SSH screening that aimed at identifying differentially expressed genes in hCG-stimulated GC [12]. When this fragment was used as a probe to screen a size-selected cDNA library of 2.5–3.5 kb generated from hCG-induced bovine GC, 26 positive bacterial colonies were obtained from a screen of 768 colonies, of which two clones were selected for plasmid DNA purification and sequencing. Results revealed that the full-length bovine *PLA2G4A* cDNA consisting of 2863 bp had been obtained, and the nucleotide sequence was submitted to Genbank (accession number AY363688). It was shown to be composed of a 5′-untranslated region (5′-UTR) of 140 bp, an open reading frame of 2247 bp (including the stop codon), and a 3′-UTR of 476 bp containing four copies of an AU-rich element (ATTTA), a motif known to contribute to short-lived mRNAs [29], as well as a polyadenylation signal (AATAAA) followed by a poly(A)$^+$ tail. The coding region of bovine PLA2G4A encodes a 749-amino acid protein, with a theoretical M_r of 85,300 and an isoelectric point of 5.2. Amino acid homology search in GenBank by PsiBlast revealed orthologous proteins with an overall identity level of 95% (equine: O77793), 94% (mouse: B39898; rabbit: AAF15299), 93% (human: NP_077734; rat: NP_598235) and 83% (chicken: NP_990754). When compared to the aforementioned species, protein domains of importance are conserved in bovine PLA2G4A, and include a calcium binding domain (C2-domain; Phe^{20}-Thr^{106}) with the essential calcium-binding residues D^{43} and D^{93}, a lysophospholipase catalytic site (PLA2-B domain; Val^{190}-Glu^{675}), and consensus phosphorylation sites (Ser^{505}, Ser^{515}, Ser^{727}).

Expression of PLA2G4A and PLA2G1B mRNAs in Granulosa Cells and CL

The expression profile of *PLA2G4A* mRNA was compared by virtual Northern analysis using GC collected from follicles obtained at different developmental stages, including SF (2–4 mm), DF at Day 5 of the estrous cycle, OF 24 h following injection of an ovulatory dose of hCG, and CL at Day 5. A single transcript estimated at 3.1 kb for *PLA2G4A* was observed and was shown to be expressed predominantly in GC of OF (data not shown). The semiquantitative RT-PCR demonstrated low expression in SF, DF, and CL, whereas a 14-fold increase in *PLA2G4A* mRNA was observed following hCG treament in GC of OF compared to DF ($P < 0.0001$; Fig. 1). In a parallel analysis, *PLA2G1B* mRNA was shown to be expressed in all samples but at a variable level; its highest expression was observed in SF, which showed a decrease of 3-fold when compared to OF ($P < 0.0001$; Fig. 1).

Regulation of PLA2G4A and PLA2G1B mRNAs in Follicles During the Periovulatory Period

Because hCG treatment clearly caused an induction of *PLA2G4A* mRNA in GC 24 h after its injection, semiquantitative RT-PCR was used to study the regulation of *PLA2G4A* and *PLA2G1B* mRNAs in ovulatory follicles isolated at different times between 0 and 24 h after hCG injection. Levels of bovine *PLA2G4A* mRNA were low, but detectable, in follicles preceding hCG treatment (0 h). An initial 16-fold upregulation of *PLA2G4A* mRNA was apparent 12 h after hCG treatment, and a maximum 45-fold increase was reached 24 h following hCG injection when compared to 0 h ($P < 0.0001$; Fig. 2A). Conversely, *PLA2G1B* mRNA expression decreased during estrus from 0 to 24 h, but the difference was not statistically significant ($P < 0.076$; Fig. 2A). The expression profile of *CYP19A1* and *PTGS2* mRNAs was investigated in order to validate the physiological status of follicles collected during the periovulatory period. Results showed that *CYP19A1* mRNA was elevated at 0 h and then declined to undetectable levels 24 h post-hCG ($P < 0.0002$; Fig. 2B). Conversely,

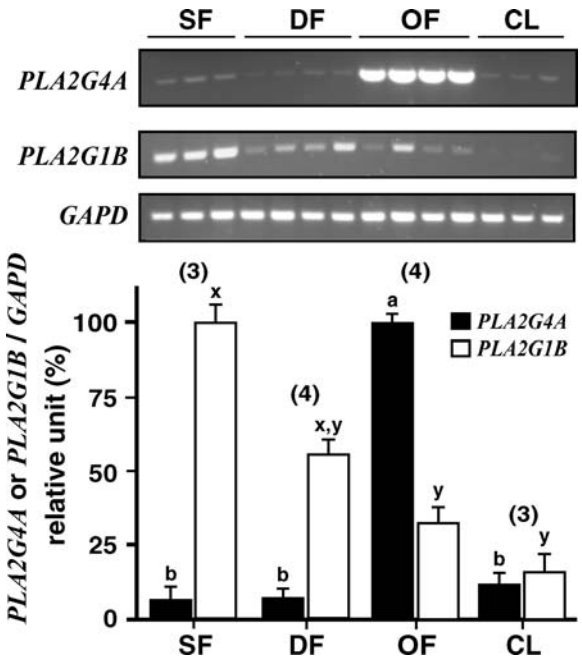

FIG. 1. Expression of *PLA2G4A* and *PLA2G1B* transcripts in bovine granulosa cells and CL. Total RNA was extracted from bovine granulosa cells collected from 2–4 mm follicles (SF) and dominant follicles (DF) at Day 5 of the estrous cycle, ovulatory follicles (OF) 24 h after injection of hCG, and corpora lutea (CL) from Day 5 of the estrous cycle, then used in mRNA expression analyses using semiquantitative RT-PCR as described in *Materials and Methods*. The control gene was *GAPD* (710 bp); it showed no significant difference in mRNA expression levels between groups. The *PLA2G4A* (960 bp) and *PLA2G1B* (437 bp) PCR fragments were normalized with their corresponding *GAPD* signals, and the results are presented as a relative change in ratio between groups. Different letters denote samples that differed significantly ($P < 0.05$) when Tukey-Kramer multiple comparison tests were performed to compare group means for a specific gene. Data are presented as means ± SEM, and the number of independent samples, i.e., animals, per group is indicated in parentheses.

PTGS2 mRNA was undetectable at 0 h, was significantly increased at 18 h, and reached a maximum level at 24 h post-hCG ($P < 0.0002$; Fig. 2B). In light of these results, we conclude that *PLA2G4A* transcript is upregulated by hCG and precedes the expression of *PTGS2*.

Regulation of PLA2G4A Protein in Ovulatory Follicles

To determine whether the increase in *PLA2G4A* mRNA in bovine ovulatory follicles after hCG treatment was associated with changes in protein level, the regulation of PLA2G4A protein was studied in GC protein extracts isolated from follicles collected between 0 and 24 h after hCG. Immunoblot analysis showed that the antibody recognized a 100 000-M_r band that was induced 18 h after hCG and reached the highest level of expression at 24 h post-hCG (Fig. 3). The 72 000 M_r band corresponding to PTGS2 was faint at 18 h and reached a maximum at 24 h (Fig. 3). The expression of GSTA1, used as a control protein, showed no significant difference between samples. To determine the cellular localization of PLA2G4A protein expression in ovulatory follicles, immunohistochemistry was performed on sections of bovine follicles isolated 0 and 24 h after hCG treatment (two follicles per time point). Immunolabeling was undetectable in GC of dominant or preovulatory follicles isolated before hCG treatment (Fig. 4, A and B). Immunoreactive staining of PLA2G4A protein was observed mainly in mural GC compared to antral GC of follicles obtained at 24 h post-hCG. Moreover, mural GC

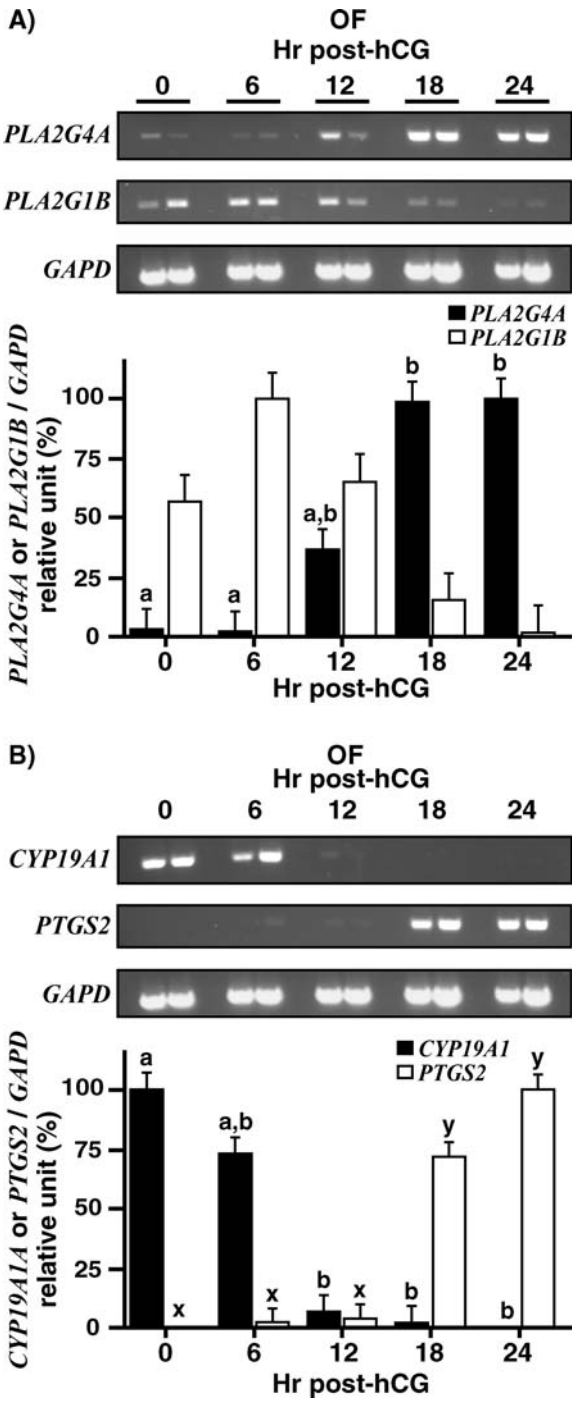

FIG. 2. Regulation of *PLA2G4A* and *PLA2G1B* transcripts by hCG in bovine follicles during the periovulatory period. Total RNA was extracted from preparations of bovine follicular wall obtained from ovulatory follicles isolated between 0 and 24 h after hCG, then used in mRNA expression analyses by semiquantitative RT-PCR as described in *Materials and Methods*. The control gene was *GAPD* (710 bp); it showed no significant differences in mRNA expression levels between groups. **A**) The *PLA2G4A* (960 bp) and *PLA2G1B* (437 bp) PCR signals were normalized with their corresponding *GAPD* signals, and the results are presented as a relative change in ratio between groups. **B**) The *CYP19A1* (520 bp) and *PTGS2* (418 bp) PCR signals were normalized with their corresponding *GAPD* signals, and the results are presented as a relative change in ratio between groups. Different letters denote samples that differed significantly ($P < 0.05$) when Tukey-Kramer multiple comparison tests were performed to compare group means for a specific gene. Data are presented as means ± SEM, and represent two distinct follicles, i.e., animals, per time point. Ten animals were utilized for ANOVA analysis followed by Tukey-Kramer multiple comparison test.

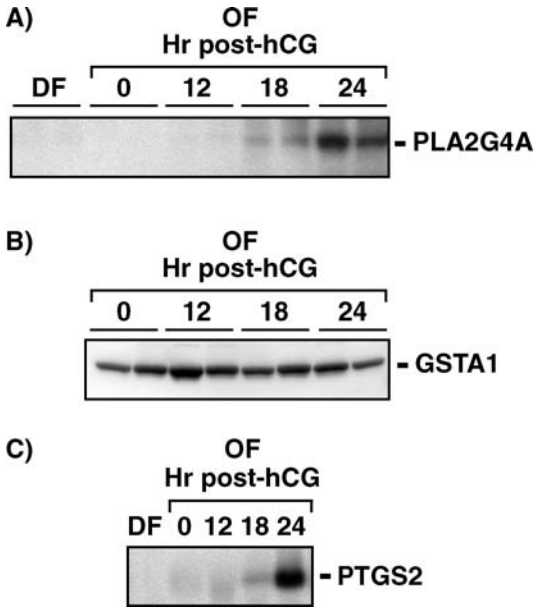

FIG. 3. Regulation of PLA2G4B and PTGS2 proteins by hCG in bovine follicles. Granulosa cells were isolated from bovine dominant follicles (DF) at Day 5 of the estrous cycle and from ovulatory follicles between 0 and 24 h after hCG treatment. Protein extracts (100 μg) were analyzed by 5%–18% gradient one-dimensional SDS-PAGE and immunoblotted as described in *Materials and Methods*. **A**) Immunoblotting was performed using a monoclonal antibody against human PLA2G4A (1:100; SC-454) and revealed a 100 000 Mr protein band. Two distinct follicles, i.e., animals, per time point are represented. **B**) Immunoblotting was performed using a rabbit anti-bovine GSTA1 antibody (1:3000) and revealed a 28 000 Mr protein band. Two distinct follicles, i.e., animals, per time point are represented. **C**) Immunoblotting was performed using a rabbit anti-human PTGS2 antibody (1:5000; MF243) and revealed a 72 000 Mr protein band. One representative follicle per time point is shown.

presented a variable signal intensity of labeling. Intracellular immunolocalization of PLA2G4A protein in GC appeared well focalized and associated with perinuclear vesicular structures (Fig. 4E). Theca cells were unstained (Fig. 4, D and E).

Regulation of PLA2G4A *Expression in Bovine Granulosa in Culture*

The LHCGR exerts its effect in GC of preovulatory follicles at least through the activation of the adenylyl cyclase/cAMP pathway. We investigated whether activation of the adenylyl cyclase/cAMP pathway would stimulate the expression of *PLA2G4A* at the mRNA and protein levels. Bovine GC were treated with 10 μM of FSK, a concentration known to stimulate the PKA pathway in these cells [28]. An increase in *PLA2G4A* mRNA expression was observed 6 h following incubation with FSK, with maximum levels of expression reached at 24 h (Fig. 5A). An increase in PLA2G4A protein expression was observed 24 h following the addition of FSK (Fig. 5B), which paralleled the maximum increase observed at the mRNA level. These results showed that *PLA2G4A* mRNA and protein expression in bovine GC are correlated with the increase in cAMP.

DISCUSSION

This study demonstrates that the process of ovulation induced by the preovulatory LH/hCG surge is preceded by the induction of *PLA2G4A* (also known as *cPLA2α*), at the mRNA and protein levels, in GC of bovine ovulatory follicles. Using primary bovine GC in culture, *PLA2G4A* mRNA and protein were also shown to be upregulated via the FSK-stimulated adenylyl cyclase/cAMP pathway. Conversely, the mRNA of the secreted phospholipase A2, *PLA2G1B* (also known as pancreatic *PLA2A*), was shown to be expressed in GC of small follicles, but its level of expression decreased in GC of preovulatory or LH/hCG-induced ovulatory follicles. In rodents, a previous study using total ovarian protein extracts showed that total PLA2 enzymatic activity changes during estrus [21]; however, no reference to the type of PLA2 family member was mentioned. A gene expression profiling study using subtractive suppression hybridization identified *PLA2G4A* as a candidate gene that may be induced in bovine GC by the LH/hCG preovulatory surge [12]. Because only a cDNA fragment was identified in the latter study, and multiple phospholipases are expressed in various tissues [30], the full-length cloning of bovine *PLA2G4A* cDNA was achieved using cDNAs derived from hCG-stimulated GC.

The bovine PLA2G4A amino acid sequence was found to be highly conserved when compared to that of other species. The Ca^{+2} binding domain (C2-domain; Phe^{20}-Thr^{106}) is responsible for the interaction of PLA2G4A to membranes and phospholipid vesicles [31, 32] for which the Ca^{+2}-binding residues D^{43} and D^{93} are essential [33, 34]. Calcium-mobilizing agonists induce AA release from membrane phospholipids by promoting the translocation of PLA2G4A from the cytosol to primarily the nuclear envelope and the endoplasmic reticulum [35–37], and by stabilizing its interaction to membranes [37, 38]. Several serines (Ser^{505}, Ser^{727}, and Ser^{515}) are also conserved in bovine PLA2G4A, and were shown to be phosphorylated in orthologous proteins [39], of which Ser^{505} was the most important for enzymatic activity [13]. However, phosphorylated PLA2G4A failed to release AA in the absence of an increase in intracellular Ca^{+2}, which indicated that phosphorylation alone is not sufficient for enzyme activation, but rather promotes its activity [38]. Thus, an increase in free intracellular Ca^{+2} concentration is the primary mechanism triggering enzymatic activation of PLA2-G4A. Interestingly, LH/hCG treatment of cultured porcine GC was shown to elicit a biphasic rise in intracellular Ca^{+2} levels via immediate intracellular Ca^{+2} mobilization and delayed transmembrane Ca^{+2} influx through the activation of phospholipase C [40, 41]. Thus, the LH/hCG preovulatory surge may activate PLA2G4A in bovine GC through increased intracellular concentration of Ca^{+2} at the time of ovulation.

In cyclic cows, injection of an ovulatory dose of hCG upregulated *PLA2G4A* mRNA expression in GC initially at 12 h post-hCG. The GC samples used in this study were also validated for mRNA expression of two genes known to be either downregulated (*CYP19A1*) or upregulated (*PTGS2*) by the preovulatory LH/hCG surge. The initial induction of *PTGS2* mRNA in GC was detected 18 h post-hCG, which corroborates previous observations in cattle [42]. Thus, in bovine GC the LH/hCG preovulatory surge induces *PLA2G4A* expression prior to *PTGS2* expression. Results obtained for *PLA2G4A* in cyclic cows corroborate and extend previous observations made in rats using either gonadotropin-primed immature or adult rats, in which PLA2G4A enzymatic activity and immunoreactivity were reported to increase at proestrus [43, 44]. Immunoblotting and immunohistochemical results obtained in GC of ovulatory follicles mirrored the induction of *PLA2G4A* mRNA by LH/hCG, whereas PLA2G4A protein was undetectable in GC of dominant or preovulatory follicles obtained at Day 5 or Day 7 of the estrous cycle. The immunostaining pattern observed in GC for PLA2G4A indicated a well-localized vesicular and perinuclear pattern, which concords with the described translocation of the PLA2G4A protein from the cytoplasm to the nuclear

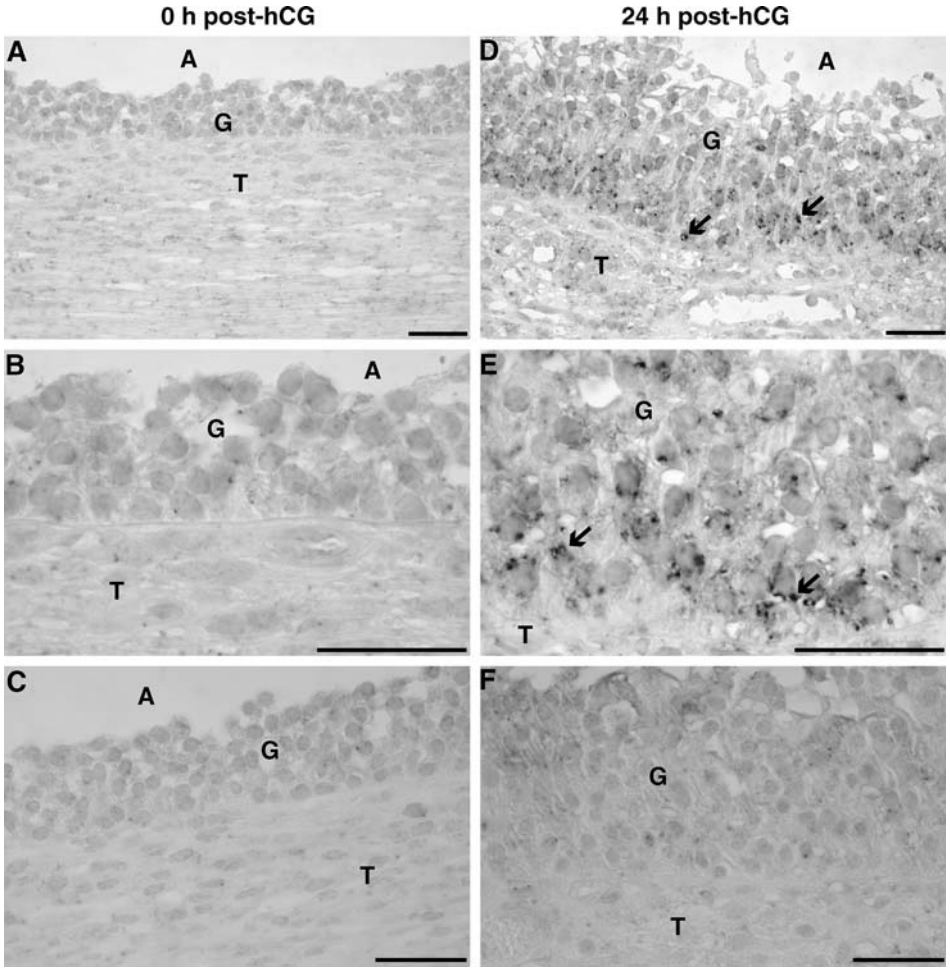

FIG. 4. Immunohistochemical localization of PLA2G4A in bovine ovulatory follicles. Immunohistochemistry was performed on formalin-fixed sections of ovulatory follicles isolated at 0 h (**A–C**) and 24 h (**D–F**) after hCG treatment, as described under *Materials and Methods*. Staining was absent when the primary antibody was omitted (**C**, **F**). Staining is undetectable in GC of preovulatory follicles obtained preceding hCG treatment (0 h; **A**, **B**). A clear induction of PLA2G4A immunoreactivity is observed in GC of ovulatory follicles obtained 24 h post-hCG (**D**, **E**). Mural GC located near the basal membrane demonstrated a stronger labeling pattern (arrows) when compared to antral GC. A, antrum; G, granulosa; T, theca. Bars = 0.05 mm.

membrane following the increase in free intracellular Ca^{+2} concentration [35–38]. The vesicular and perinuclear localization of activated PLA2G4A also match the described perinuclear localization of PTGS2 [45]. Furthermore, we observed that mural GC stained more intensely for PLA2G4A when compared to antral GC. Interestingly, it is known that mural and antral GC differ in the level of LHCGR, with higher levels of expression in mural GC [46, 47]. Thus, the higher expression of LHCGR by mural GC could also explain the higher induction of PLA2G4A in these cells, thereby allowing an effective diffusion of released PG to the blood vessels of the theca layer.

PLA2G4A preferentially releases AA from phospholipids, thereby providing the upstream substrate for PTGS2 to produce PG [48]. In the bovine ovarian follicle, *PTGS1* is constitutively expressed in the theca cells but absent in GC, whereas *PTGS2* is inducible by LH/hCG only in GC [42]. Following the LH/hCG surge, the expression of *PLA2G4A* mRNA in GC precedes that of *PTGS2*; both enzymes are temporally regulated in a similar fashion, and once activated, they colocalize in perinuclear vesicular structures. These observations underscore that the two enzymes are metabolically coupled in GC during ovulation to ensure PG synthesis as required for ovulation and oocyte maturation. Recently, *PLA2G4A* and *PTGS2* mRNAs were also observed to be coinduced in the proresolving phase of the acute inflammatory process [49]. Because ovulation is considered to be an inflammatory reaction [2], the expression of PLA2GA4 may contribute to the release of the AA substrate necessary for the release of PTGS2-derived proinflammatory mediators such as PGs and leukotrienes. Interestingly, in the acute inflammatory reaction, interleukin-1β (IL1B) stimulates the expression of PLA2G4A [49]. Similarly, IL1B stimulated the expression of PLA2G4A in cultured rat GC [50, 51] whereas LH stimulated the expression of IL1B in human GC [52]. Furthermore, the

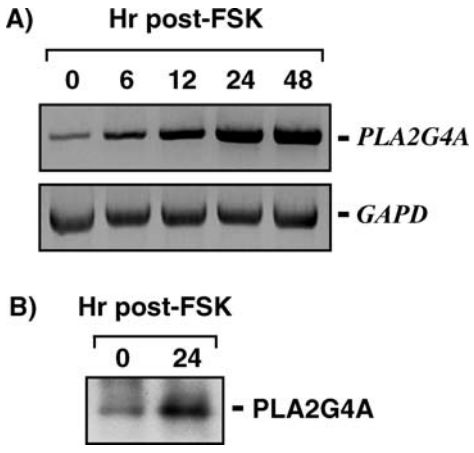

FIG. 5. Regulation of PLA2G4A expression in bovine granulosa cells in culture. Bovine GC were cultured as described under materials and methods in the presence of 10 μM FSK, and cells were collected at different time points (0–48 h post-FSK) for extraction of total RNA and protein. Representative results obtained from two experiments are presented. **A**) Results show that FSK stimulates *PLA2G4A* mRNA expression by 6 h, which expression then reaches a maximum level at 24 h post-FSK. No difference in *GAPD* mRNA was detected. **B**) PLA2G4A protein was also induced 24 h post-FSK.

receptor IL1R1 is synthesized in GC of different species [53]. LH and IL1B were shown to synergistically stimulate PG synthesis by GC [54]. Collectively, these results strongly support a model in which LH/hCG coinduces the expression of PLA2G4A and PTGS2 in GC of ovulatory follicles, as well as the expression of IL1B. The latter acts as a paracrine factor that synergizes with LH/hCG signaling to promote maximal expression and activity of PLA2G4A and PTGS2.

We have demonstrated that FSK treatment stimulated *PLA2G4A* mRNA and protein expression in bovine GC in vitro. Using the same bovine GC model, we previously showed that FSK also induced the expression of PTGS2 [28]. In GC, increase in cAMP leads to the activation of signaling mechanisms such as PKA and guanine nucleotide-exchange factors (also known as exchange protein directly activated by cAMP or EPAC) [55, 56]. Interestingly, the promoter sequence of human *PLA2G4A* contains a cAMP-responsive element (CRE), which suggests that CRE-binding proteins may promote the expression of *PLA2G4A* mRNA in GC.

We investigated whether other PLA2 isoforms were expressed by bovine GC. Because the availability of cDNA sequences for different *PLA2* isoforms is limited in the bovine species, the expression of a secreted form of *PLA2, PLA2G1B,* was investigated. The expression of *PLA2G1B* mRNA was higher in GC of small follicles and reached the lowest level in GC of hCG-stimulated ovulatory follicles. Thus, expression of *PLA2G1B* mRNA in GC was inversely related to *PLA2G4A* during ovulation. We concluded that PLA2G1B does not supply AA to PTGS2 during ovulation.

Ovulation and oocyte maturation were shown to be inhibited in female mice null for *PTGS2,* suggesting an essential role for PG in these processes [57]. Conversely, *PLA2G4A*-null mice showed a decrease in fertility that was associated mainly with modifications at the embryo uterine interaction level, but ovulation was modestly reduced [58, 59]. However, we have clearly shown an induction of *PLA2G4A* mRNA and protein in bovine GC by LH/hCG, which supports a role for PLA2G4A in providing AA substrate for PTGS2. Interestingly, intraovarian bursal injection of arachidonyl trifluoromethyl ketone, a specific PLA2G4A inhibitor, significantly decreased ovulation and total ovarian PGE2 synthesis in rats [44], which underscores a role for PLA2G4A activity in the release of AA necessary for PTGS2. Discrepancies between our observations made in the bovine species and in the *PLA2G4A* knockout mouse model suggest that compensatory mechanisms may develop with other PLA2 isoforms contributing to the release of AA substrate. Thus, the temporal expression of other PLA2 isoforms should be investigated, as well as their relative contribution to ovulation.

Ovulation is associated with an LH/hCG-dependent induction of PLA2G4A in granulosa cells following activation by the LHCGR of the adenylyl cyclase/cAMP pathway.

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