BIOLOGY OF REPRODUCTION **77,** 626–635 (2007)
Published online before print 20 June 2007.
DOI 10.1095/biolreprod.106.054957

Identification of Calcitonin Expression in the Chicken Ovary: Influence of Follicular Maturation and Ovarian Steroids[1]

Susan M. Krzysik-Walker, Olga M. Ocón-Grove, Sreenivasa B. Maddineni, Gilbert L. Hendricks III, and Ramesh Ramachandran[2]

Department of Poultry Science, Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT

Calcitonin (CALCA), a hormone primarily known for its role in calcium homeostasis, has recently been linked to reproduction, specifically as a marker for embryo implantation in the uterus. Although CALCA expression has been documented in several tissues, there has been no report of production of CALCA in the ovary of any vertebrate species. We hypothesized that the *Calca* gene is expressed in the chicken ovary, and its expression will be altered by follicular maturation or gonadal steroid administration. Using RT-PCR, we detected *Calca* mRNA and the calcitonin receptor (*Calcr*) mRNA in the granulosa and theca layers of preovulatory and prehierarchial follicles. Both CALCA and *Calca* mRNA were localized in granulosa and thecal cells by confocal microscopy. Using quantitative PCR analysis, F1 follicle granulosa layer was found to contain significantly greater *Calca* mRNA and *Calcr* mRNA levels compared with those of any other preovulatory or prehierarchial follicle. The granulosa layer contained relatively greater *Calca* and *Calcr* mRNA levels compared with the thecal layer in both prehierarchial and preovulatory follicles. Progesterone (P_4) treatment of sexually immature chickens resulted in a significantly greater abundance of ovarian *Calca* mRNA, whereas estradiol (E_2) or P_4 + E_2 treatment significantly reduced ovarian *Calca* mRNA quantity. Treatment of prehierarchial follicular granulosa cells in vitro with CALCA significantly decreased FSH-stimulated cellular viability. Collectively, our results indicate that follicular maturation and gonadal steroids influence *Calca* and *Calcr* gene expression in the chicken ovary. We conclude that ovarian CALCA is possibly involved in regulating follicular maturation in the chicken ovary.

estradiol, follicular development, granulosa cells, progesterone, theca cells

INTRODUCTION

Calcitonin (CALCA) is a peptide hormone that is primarily associated with maintaining calcium homeostasis. Synthesized as procalcitonin in the thyroid gland of mammals or the ultimobranchial gland of avian species, CALCA is secreted in response to hypercalciemia [1]. To maintain constant levels of circulating calcium ions, CALCA inhibits osteoclastic resorption of calcium from bone but increases excretion of calcium ion in the kidneys [2, 3]. Recent studies have identified numerous nonthyroidal sources of CALCA, which include the thymus, jejunum, lung, urinary bladder [4], prostate [5], endometrium [6], pituitary gland [7], and mammary glands [8, 9]. Although CALCA is not well characterized in most tissues, the physiologic role of CALCA has been well documented in the pituitary gland and endometrium, wherein CALCA has been shown to inhibit prolactin secretion and favor embryo implantation, respectively [6, 10].

Numerous studies have suggested a role for CALCA in female reproduction. Circulating CALCA levels have previously been shown to rise significantly just before ovulation in the rainbow trout, eel, and rat [11–13]. More recent studies, however, have focused on CALCA's involvement in blastocyst implantation. In cycling rats, both *Calca* mRNA and CALCA are expressed in the glandular tubular epithelial cells of the endometrium, with significant amounts of CALCA secreted into the uterine lumen on Days 4 and 5 of gestation immediately preceding implantation [14]. An increase in endometrial *Calca* gene expression has been documented during the progesterone-dominant phase of the estrus or menstrual cycle, in which the uterus is most receptive to embryonic implantation in rats, baboon, and humans [6, 15, 16]. A transient expression of *Calca* in the preimplantation phase of the uterus appears to be critical for blastocyst implantation, since attenuation of *Calca* expression by administering antisense oligonucleotides is accompanied by a severe impairment in implantation of embryos. [17]. In addition, both estrogen (E_2) and progesterone (P_4) appear to play a major role in the expression of endometrial *Calca*. Administration of E_2 or P_4 was found to decrease and increase endometrial *Calca* gene expression, respectively [6, 14].

Despite numerous studies describing *Calca* expression in several tissues, there is no clear evidence to suggest that *Calca* is expressed within the ovary itself. While exploring *Calca* expression in various chicken tissues, we serendipitously detected *Calca* cDNA expression in the chicken ovary. Based on this observation, we desired to further explore and characterize the expression of CALCA in the chicken ovary and determine how follicular maturation and gonadal steroids may alter ovarian *Calca* expression. We present novel evidence that both the *Calca* mRNA and CALCA peptide are expressed in the granulosa and theca cells of the ovarian follicles. We also provide novel evidence on the identification of the CALCA receptor (*Calcr*) mRNA within both the granulosa and theca cell layers. Furthermore, we show that follicular maturation significantly affects both *Calca* and *Calcr* mRNA expression within the granulosa layer, whereas CALCA influences the viability of prehierarchial follicle granulosa cells cultured in the presence of FSH.

[1]Supported in part by National Research Initiative Competitive Grant no. 2004-35203-14755 from the US Department of Agriculture Cooperative State Research, Education, and Extension Service to R.R.
[2]Correspondence: Ramesh Ramachandran, The Pennsylvania State University, Department of Poultry Science, 213 Henning Bldg., University Park, PA 16802. FAX: 814 865 5691; e-mail: RameshR@psu.edu

Received: 25 June 2006.
First decision: 4 August 2006.
Accepted: 18 June 2007.

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

MATERIALS AND METHODS

Reagents

Trizol and RNeasy kits used to isolate RNA were obtained from Invitrogen Corp. (Carlsbad, CA) and Qiagen, Inc. (Valencia, CA), respectively. M-MuLV reverse transcriptase and Taq polymerase used for RT-PCR were purchased from New England Biolabs (Beverly, MA). Additional PCR and RT materials (Platinum SYBR Green qPCR Super Mix-UDG, RNAseOut) were obtained from Invitrogen. Digoxigenin-labeled nucleotides, dinucleotide triphosphate mix, Sp6 polymerase, T7 polymerase, and antidigoxigenin antibody were purchased from Roche Applied Sciences (Indianapolis, IN). Estradiol 17-β and progesterone were purchased from Sigma-Aldrich (St. Louis, MO). Anti-eel CALCA antibody was purchased from Peninsula Laboratories (Belmont, CA). Biotinylated anti-rabbit immunoglobulin G (IgG) and streptavidin-Alexa 488 were purchased from Vector Laboratories (Burlingame, CA), and TOPRO-3 was purchased from Invitrogen. Chicken CALCA was purchased from Bachem (Torrance, CA), and recombinant human FSH was purchased from the National Hormone and Peptide Program (Torrance, CA). The CellTiter-Blue Cell Viability Assay was purchased from Promega (Madison, WI).

Animals

A commercial strain of Leghorn chickens (Hyline W36 strain) was maintained at the Poultry Research and Extension Center at Pennsylvania State University (University Park, PA). The chickens were provided with 16L:8D photoperiod and were provided with water and feed ad libitum. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee-approved protocol.

Collection of Ovary/Separation of Granulosa or Theca Layer

Approximately 4–6 h prior to ovulation, chickens (n = 6; Hyline W36 strain; 60 wk old) were killed by decapitation with a guillotine. Prior to killing, the presence of a hard-shelled egg in the shell gland was confirmed by cloacal examination. Immediately following killing, the ovarian follicles were separated from the ovary and categorized based on their diameter into preovulatory follicles (F1–F6) and prehierarchial follicles (6–8 mm and 3–5 mm). The separated follicles were placed into ice-cold 0.75% saline. The granulosa and theca cell layers from each preovulatory follicle were separated using a dissection microscope following the method described previously [18], snap frozen in liquid nitrogen, and stored at −80°C until processed. Granulosa and thecal cell layers obtained from two to three prehierarchial follicles within each category (3–5 mm and 6–8 mm) were pooled from each animal.

Calca *and* Calcr *RT-PCR*

Granulosa and thecal cell layers from preovulatory and prehierarchial follicles, as well as other tissues (adipose, liver, hypothalamus, skeletal muscle, kidney, spleen, total ovary, chondrocyte, pituitary, and blood), were collected from sexually mature female chickens. Total RNA was extracted from these tissues using Trizol (Invitrogen) and/or the RNeasy kit (Qiagen, Valencia, CA). Following on-column DNAse I (Qiagen) treatment, first-strand cDNA was synthesized by reverse transcribing 1 μg total RNA using $d(T)_{30}$A/G/C A/G/C/T, 2U M-MLV reverse transcriptase (New England BioLabs) in a 20- to 50-μl reaction. Approximately 100 ng single-stranded cDNA was used as template to amplify a 204-bp product of *Calca* cDNA using CT1Fwd and CT1Rev primers (Table 1) or a 495-bp product of *Calcr* cDNA using CTR1Fwd and CTR1Rev primers (Table 1). A touch-down PCR was performed using the following thermocycle: 94°C for 1 min, 30 cycles of 94°C for 5 sec and 72°C–68°C for 3 min. Annealing and primer extension were done at 72°C, 70°C, and 68°C during 1–5, 6–10, and 11–30 cycles, respectively. The PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining for visualization. For negative controls, reverse transcription reactions using 1 μg total RNA from each tissue/follicle with no reverse transcriptase (−RT) were used as a template in place of reverse transcription reactions that contained reverse transcriptase (+RT). In addition, total RNA extracted from blood cells was subjected to RT-PCR to rule out blood contamination as a source of *Calca* mRNA. Using another set of primers (CT2Fwd and CT2Rev; Table 1), a 417-bp, full-length *Calca* cDNA was amplified with the F1 follicle granulosa cell cDNA as a template. The resultant products were sequenced (Davis Sequencing, San Diego, CA) to confirm the authenticity of *Calca* and *Calcr* cDNAs.

Cellular Localization of Calca *mRNA by In Situ Hybridization Histochemistry in Ovary*

Preparation of digoxigenin-labeled Calca *cRNA probe.* A partial 345-bp-long chicken *Calca* cDNA was amplified from a chicken pituitary cDNA library and was subcloned into pGEM-T Easy vector (Promega, Madison, WI). The resultant plasmid was linearized using *Pst*I or *Nco*I restriction endonucleases (New England BioLabs) and gel purified. Antisense and sense strands of *Calca* cDNA were transcribed using digoxigenin-labeled nucleotide (Roche Biochemicals) with T7 polymerase and Sp6 polymerase, respectively.

In situ hybridization histochemistry. A small piece of chicken ovary was fixed by immersion in 4% paraformaldehyde for 1 h at room temperature. Following fixation, the tissue was washed three times in PBS for 15 min each, dehydrated, cleared with xylene, and infiltrated with paraffin. Ovary tissue sections (10 μm) were cut using a rotary microtome (Microm, Walldorf, Germany), and serial sections were mounted on Superfrost Plus glass slides (VWR, West Chester, PA). Tissue sections were deparaffinized, hydrated, and treated with 0.2 N hydrochloric acid. Slides were rinsed in 0.3% Triton X-100 and treated with proteinase K (10 μg/ml; Roche Biochemicals) in 0.1 M Tris HCl containing 50 mM EDTA for 20 min. Tissue sections were then treated in 0.2% glycine for 5 min, fixed in 3.7% paraformaldehyde, and acetylated with 0.25% acetic anhydride and 0.1 M triethanolamine. The slides were then incubated in prehybridization buffer (50% formamide in 2× SSC) for 90 min at 37°C. Digoxigenin-labeled *Calca* cRNA probe (antisense, or sense strand for a negative control) were diluted in hybridization buffer (10 mM Tris-HCL, 12.5% Denhardt solution, 50% formamide, 0.5% SDS, 2× SSC, 0.73 mg/ml yeast tRNA, and 0.36 mg/ml salmon sperm) and applied to the slides under a coverslip. The slides were incubated in a humidified chamber at 45°C overnight. Afterwards, the slides were treated with RNAse (20 μg/ml; Gibco) in 10 mM Tris-HCl, 5 mM EDTA, and 0.3 M NaCl for 30 min at 37°C. Slides were then washed twice in 2× SSC for 15 min each time at room temperature, twice in 0.1× SSC for 5 min each, and twice in 0.1× SSC for 30 min each at 42°C. Next, the slides were washed in Tris-buffered saline (TBS) containing 0.1% Triton X-100 (Sigma) for 10 min, and treated with 1× blocking buffer (Roche Biochemicals) in TBS for 30 min. A monoclonal anti-digoxigenin

TABLE 1. The nucleotide sequences of the primers used to amplify *Calca, Actb,* and *Calcr*.

Name	Sequence	GenBank accession no.	Product length (bp)
Calca			
CT1Fwd	5′-AGTCAGACCTGGCTTGGAGTCCATCA-3′	X03012	204
CT1Rev	5′-TGAGACAGTTTGCCCAGCACACAAGT-3′		
CT2Fwd	5′-ATGGTCATGCTGAAGATTTCATCTTTCCT-3′	XM_420997	417
CT2Rev	5′-CTAGTTGTTTCCTAGGGTTTCCCCATAGTT-3′		
Actb			
BAFwd	5′-CTGGCACCTAGCACAATGAA-3′	L08165	123
BARev	5′-CTGCTTGCTGATCCACATCT-3′		
Calcr			
CTR1Fwd	5′-CATGCAGTTGCAAGAGCCAAATACTTCAAT-3′	XM_425985	495
CTR1Rev	5′-GTACAGGTAGACAGGGACCTCTGTGATGGA-3′		
CTR2Fwd	5′-TGGCAACTATATTCTGCTTCTTCA-3′	XM_425985	136
CTR2Rev	5′-GACGTTGCTGTGTAGGAGGTAGAT-3′		

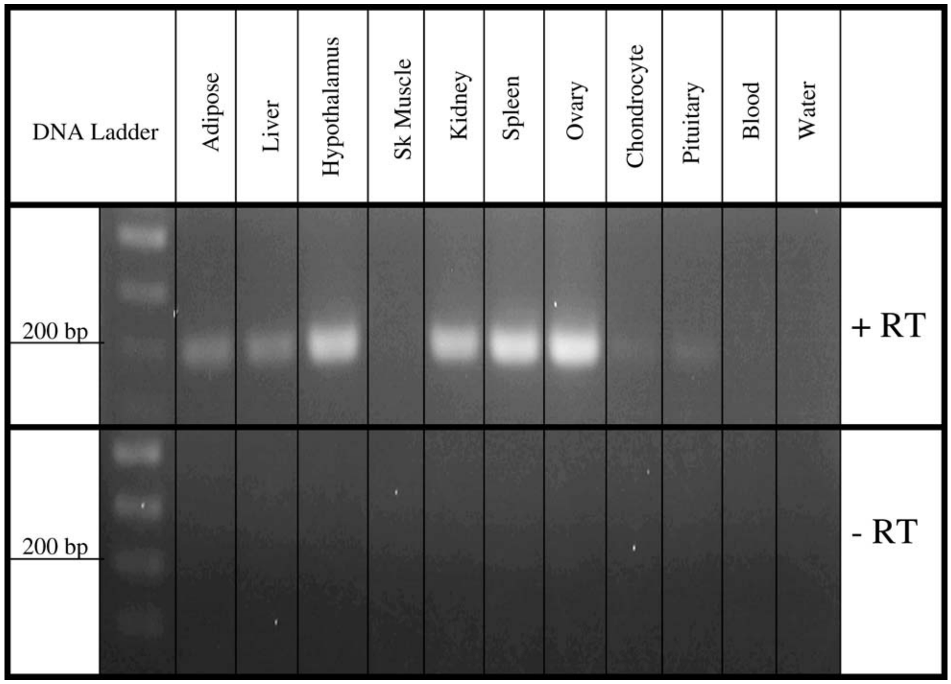

FIG. 1. RT-PCR analysis of *Calca* gene expression in various tissues of the chicken. Approximately 100 ng cDNA (+RT) was used as template to amplify a 204-bp chicken *Calca* cDNA. Contamination controls consisted of RNA from each tissue without reverse transcriptase (-) or substitution of water for the cDNA template. SkM, skeletal muscle.

antibody (1:100; Roche Biochemicals) was applied to all slides and incubated at 4°C overnight. Following three washes in TBS for 5 min each, slides were treated with biotinylated anti-mouse IgG made in goat (1:400; Vector Laboratories) for 1 h at room temperature. Slides were washed three times in TBS and incubated with streptavidin-Alexa 488 (1:100; Invitrogen) for 1 h at room temperature. Following three more washes in TBS, slides were mounted with ProLong Gold Antifade Reagent and TOPRO-3 (1:500; Invitrogen). Green (*Calca*) fluorescent cells and their nuclei (red) were visualized by exciting the flourophores with respective lasers using an Olympus Fluoview 300 Confocal Laser Scanning Microscope (Olympus, Center Valley, PA).

Immunohistochemical Detection of Ovarian CALCA

Paraffin-embedded chicken ovary tissue sections were prepared as described above. Tissue sections were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA), hydrated in decreasing concentrations of ethanol in water, and rinsed in TBS. Following a rinse in TBS containing 0.1% Triton X-100 (TBSX), slides were treated with 1% goat serum in TBSX for 30 min. Slides were then treated with affinity-purified rabbit anti-eel CALCA (Peninsula Laboratories, Belmont, CA), at a concentration of 40 μg/ml overnight at 4°C. Due to the unavailability of an anti-chicken CALCA, we selected anti-eel CALCA based on the close sequence homology (94%) as well as previous studies that used a similar antibody to identify immunoreactive cells in chicken tissue [19]. After rinsing with TBS, biotinylated goat anti-rabbit IgG (1:400; Vector Laboratories) was applied to the slides and allowed to incubate for 1 h, followed by rinsing in TBS. Sections were then incubated with streptavidin-Alexa 488 (Invitrogen) at a concentration of 1:100 for 1 h. Following three washes in TBS, sections were mounted with ProLong Gold antifade and TOPRO-3 (1:500; Invitrogen). As a negative control, nonimmune rabbit IgG (Vector Laboratories) was substituted in place of the primary antibody. Green (CALCA) fluorescent cells and their nuclei (red) were visualized by exciting the flourophores with respective lasers using an Olympus Fluoview 300 Confocal Laser Scanning Microscope.

Effect of Follicular Maturation on Calca *and* Calcr *mRNA Quantity*

Total RNA extracted from the preovulatory follicles (F1, F3, and F6) and prehierarchial follicles (3–5 mm) was subjected to quantitative PCR for determination of *Calca* and *Calcr* mRNA abundances. Total RNA (1 μg) was reverse transcribed using $d(T)_{30}$A/G/C A/G/C/T, 2U M-MLV reverse transcriptase in a 20-μl reaction. Chicken *Calca* mRNA, *Calcr* mRNA, and *Actb* mRNA were quantified utilizing 5–12 μl of the reverse transcription reaction (equivalent to 100–240 ng single-stranded cDNA) as template in the real-time quantitative PCR analysis. A 204-bp product for chicken *Calca* cDNA was amplified using the CT1Fwd and CT1Rev primers (Table 1), whereas a 136-bp product of chicken *Calcr* was amplified using CTR2Fwd and CTR2Rev primers (Table 1). Similarly, a 123-bp product of chicken *Actb* was amplified using BAFwd and BARev primers (Table 1). The real-time quantitative PCR consisted of 1× Platinum SYBR Green qPCR Super Mix-UDG and 300 nM of forward and reverse primers. The reactions were carried out in the DNA Engine Opticon II (MJ Research) with the following thermocycle parameters: 50°C for 2 min and 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. At the end of amplification, a melting curve analysis was done by heating the PCR products from 65°C–95°C, held for 15 sec at increments of 0.2°C, and the fluorescence was detected to confirm the presence of a single amplification product. Tissue samples from each animal were run in duplicate to obtain average C_T values for *Calca* mRNA, *Calcr* mRNA, and *Actb* mRNA. The log-linear threshold values (C_T) during the exponential phase of the PCR for *Calca* mRNA or *Calcr* mRNA were subtracted from those of *Actb* mRNA. *Calca* mRNA quantity and *Calcr* mRNA quantity were expressed as a proportion of *Actb* mRNA quantity following $2^{-\Delta\Delta C_T}$ method for converting log-linear C_T values to linear term [20]. The relative amounts of *Calca* mRNA or *Calcr* mRNA in the follicles were then compared.

Effect of Ovarian Steroids on Ovarian Calca *mRNA Quantity*

Sexually immature female chickens (16 wk old; n = 7) were injected intramuscularly with peanut oil containing estradiol-17β (E_2; 0.5 mg/kg body weight; four injections on alternate days [21]), progesterone (P_4; 0.17 mg/kg body weight per day for 7 consecutive days [22]), estradiol and progesterone together ($E_2 + P_4$) at the above dosages, or no steroids (negative control). Seven days after the first dose, the chickens were killed by decapitation, and the oviduct was isolated (infundibulum to shell gland) and weighed to confirm the efficacy of estradiol and/or progesterone treatments. Ovaries were isolated and snap frozen in liquid nitrogen. Total RNA from the ovaries of each chicken was extracted and subjected to quantitative PCR analysis for the determination of *Calca* mRNA and *Actb* mRNA, as described above. The amount of *Calca* mRNA is expressed as a proportion to *Actb* mRNA and compared among the treatment groups.

Effect of Chicken CALCA on Granulosa Cell Viability

Granulosa cell isolation from prehierarchial follicles. Approximately 2 h prior to ovulation, chickens (Hyline W36 strain; 70 wk old) were killed by decapitation with a guillotine. Prior to killing, the presence of a hard-shelled egg in the shell gland was confirmed by cloacal examination. The granulosa layers of 10–12 prehierarchial follicles (6–8 mm in size) were removed from two hens each, pooled, and dispersed in 0.3% collagenase as previously

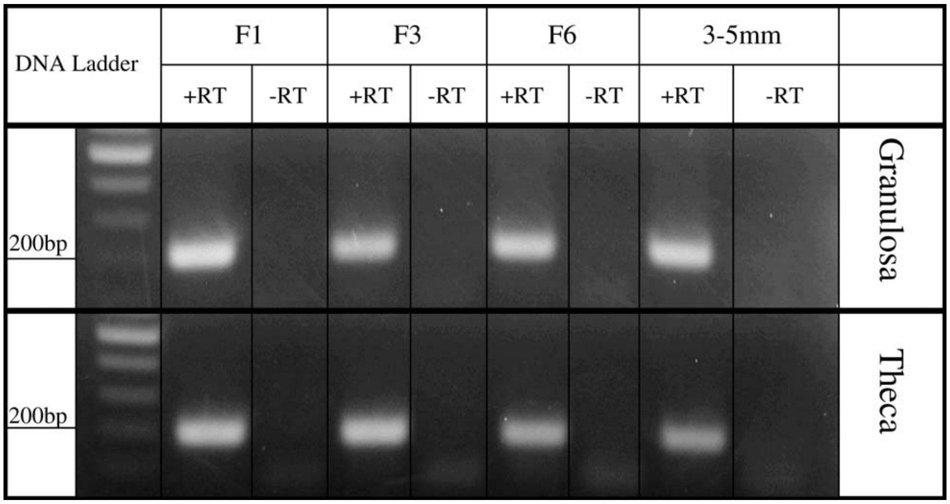

FIG. 2. RT-PCR analysis of *Calca* gene expression in granulosa and thecal cells layers of preovulatory (F1, F3, and F6) and prehierarchial (3–5 mm) follicles. Approximately 100 ng cDNA (+RT) was used as a template to amplify a 204-bp product of chicken *Calca*. Contamination controls consisted of RNA from each tissue without reverse transcriptase (-).

described [23]. Cell viability and number were ascertained by trypan blue exclusion method.

Cell viability assay. Cell viability was measured by the CellTiter-Blue Cell Viability Assay following the manufacturer's protocol. The assay determines the metabolic activity of cells based on the ability of living cells to reduce the nonfluorescent reporter compound resazurin into a fluorescent resorufin. Approximately 15 000 cells were seeded into each well of a 96-well black wall plate (PerkinElmer, Waltham, MA) coated with 0.1% gelatin. After culturing for 6 h in 100 μl culture media (M199 with Hanks salts, 0.2% BSA, 2.5% fetal bovine serum, 0.2% α-D(+) glucose, 0.01% trypsin inhibitor (lima bean, Type II-L), 1% antibiotic-antimycotic solution) at 40°C with 5% CO_2, media was removed, and treatments applied. Chicken CALCA (0, 10^{-6}, 10^{-8}, 10^{-10} M) with or without 100 ng/ml recombinant human FSH (FSH) dissolved in 100 μl culture medium was applied, and the plates were incubated for 12 or 24 h. All treatments were done in triplicate. At the end of 12 or 24 h of incubation, 20 μl CellTiter-Blue was added to each well, and incubation continued for an additional 1 h. Following incubation, resorufin fluorescence($544_{Ex}/590_{Em}$) was measured using Victor[3] 1420 Multilable Counter (PerkinElmer), and cell viability was calculated as a percentage of viability recorded from vehicle-treated cells. The experiment was repeated four times (n = 4).

Statistical Analyses

All analyses were completed by analysis of variance (ANOVA) using the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Differences between individual means were partitioned further by performing pair-wise comparisons of least square means. A probability level of $P \leq 0.05$ was considered statistically significant. For analysis of real-time PCR results, relative *Calca* mRNA to *Actb* mRNA or *Calcr* mRNA to *Actb* mRNA quantities were first converted from log-linear to linear terms. Data on the effect of gonadal steroid treatments on ovarian *Calca* mRNA abundance are expressed as fold differences compared with the vehicle treatment, whereas data on cell viability are expressed as percentage viable compared with the vehicle treatment for each time point. All data are represented as ± SEM.

RESULTS

Detection of Calca *and* Calcr *mRNA by RT-PCR*

A 204-bp partial *Calca* cDNA corresponding to nucleotides 48–251 (GenBank accession no. X03012) of the ultimobranchial gland *Calca* gene was detected in total RNA extracted from sexually mature female chicken adipose, liver, hypothalamus, kidney, spleen, and ovary (Fig. 1). A very low level of *Calca* cDNA was amplified from the pituitary gland RNA, possibly reflecting the reproductive status of the hen, as we earlier found that sexually mature female chickens had lower levels of *Calca* mRNA in the pituitary gland compared with sexually immature chickens [24]. To further characterize chicken ovarian *Calca* cDNA expression, RT-PCR was performed using total RNA extracted from the granulosa and theca cell layers of preovulatory follicles (F1, F3, and F6) and prehierarchial follicles (3–5 mm). Both granulosa and theca cell layers in all of the follicles studied expressed *Calca* cDNA (Fig. 2). The use of either water in place of cDNA or RNA that was not reverse transcribed failed to produce any PCR product (Figs. 1 and 2), confirming the absence of genomic DNA contamination. Likewise, the blood cDNA failed to amplify any PCR product, thereby eliminating blood contamination as the source for *Calca* mRNA. Furthermore, RNA or cDNA sample integrity was confirmed by successfully amplifying a fragment of glyceraldehyde-3-phosphate dehydrogenase cDNA (data not shown). To determine whether ovarian *Calca* mRNA shares similarity with that produced by the ultimobranchial gland, the primary source of CALCA in avians, the full-length *Calca* cDNA was amplified from the F1 follicle granulosa cell RNA. We found that the nucleotide sequence of the 417-bp

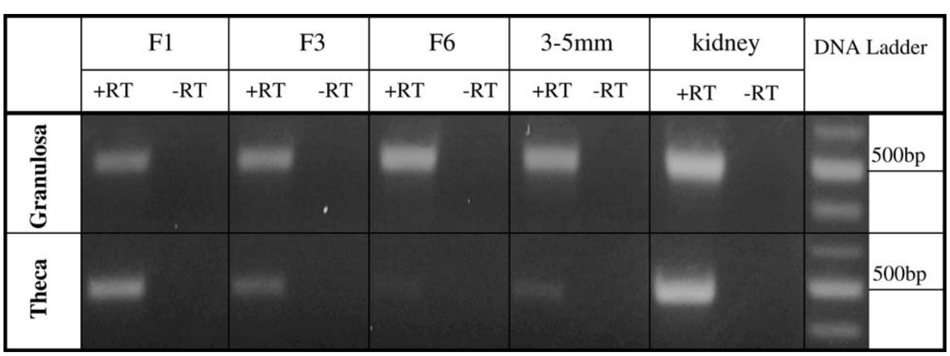

FIG. 3. RT-PCR analysis of *Calcr* gene expression in granulosa and thecal cells layers of preovulatory (F1, F3, and F6) and prehierarchial (3–5 mm) follicles, as well as from the kidney. Approximately 100 ng cDNA (+RT) was used as a template to amplify a 495-bp product of chicken *Calcr*. Contamination controls consisted of RNA from each tissue without reverse transcriptase (-).

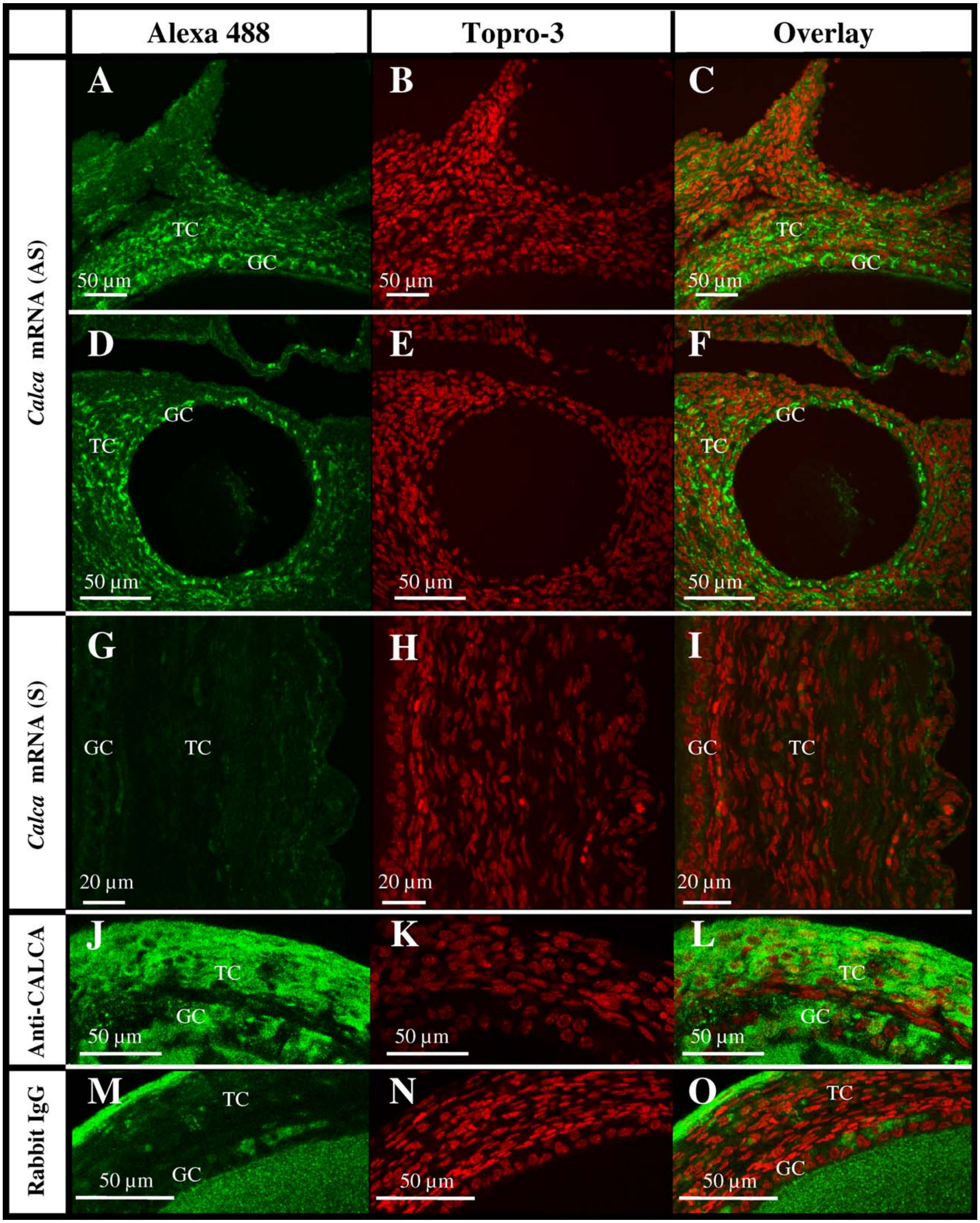

FIG. 4. Confocal photomicrographs of the chicken ovary showing *Calca* mRNA and CALCA-immunoreactive (ir) cells in the follicles. The chicken ovary tissue sections were subjected to in situ hybridization (**A**–**I**) or immunohistochemistry (**J**–**O**) to detect *Calca* mRNA and CALCA, respectively. *Calca* mRNA-expressing cells, detected by hybridizing with *Calca* antisense riboprobe, were localized to both the granulosa (GC) and theca (TC) cells (**A**–**F**). **G**–**K**) Tissue sections hybridized with sense *Calca* riboprobe as negative control. CALCA-ir cells were detected by immunohistochemistry using rabbit anti-CALCA IgG (**J**–**L**) or rabbit IgG (**M**–**O**). Green (CALCA) and red (nuclear staining) fluorescent cells were visualized using Alexa488 and TOPRO-3, respectively, using a laser confocal microscope.

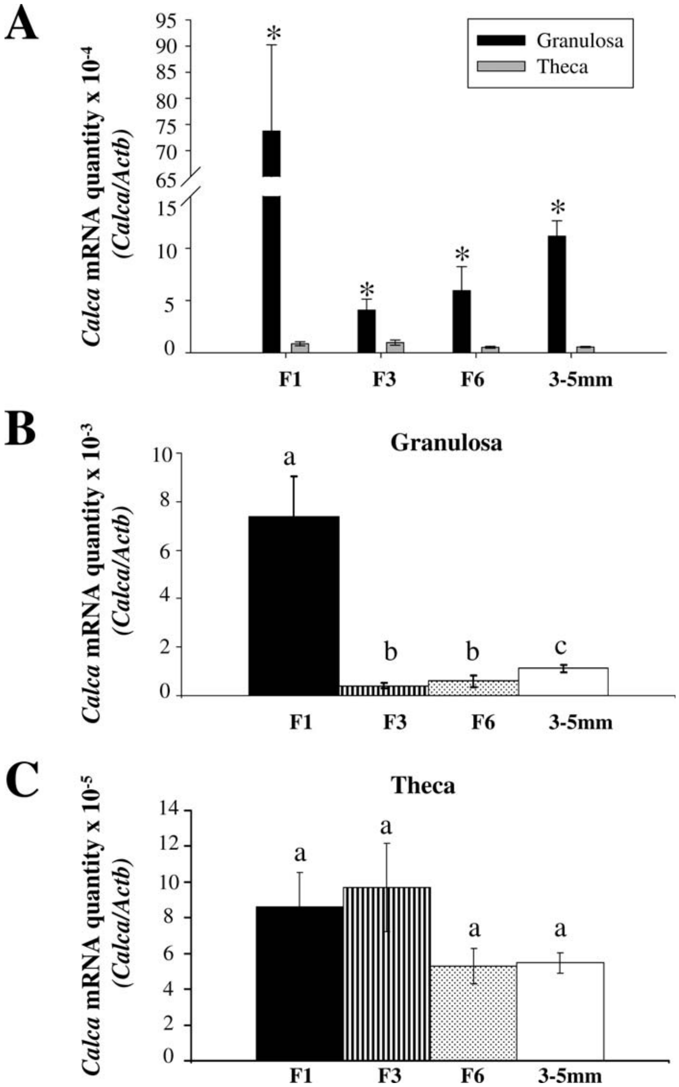

FIG. 5. **A**–**C**) *Calca* mRNA abundance in the granulosa and theca cells of preovulatory (F1, F3, and F6) and prehierarchial (3–5 mm) follicles as measured by real-time quantitative PCR. **A**) A comparison of *Calca* mRNA abundance between granulosa and thecal cell layers of preovulatory and prehierarchial follicles. An asterisk above each set of bars indicates significant differences at $P < 0.05$. **B**, **C**) A comparison of *Calca* mRNA abundance within the granulosa and theca layers. Different letters above each bar indicate significant difference at $P < 0.05$. Data in **A**–**C** represent mean ± standard error of the mean (n = 5 to 6).

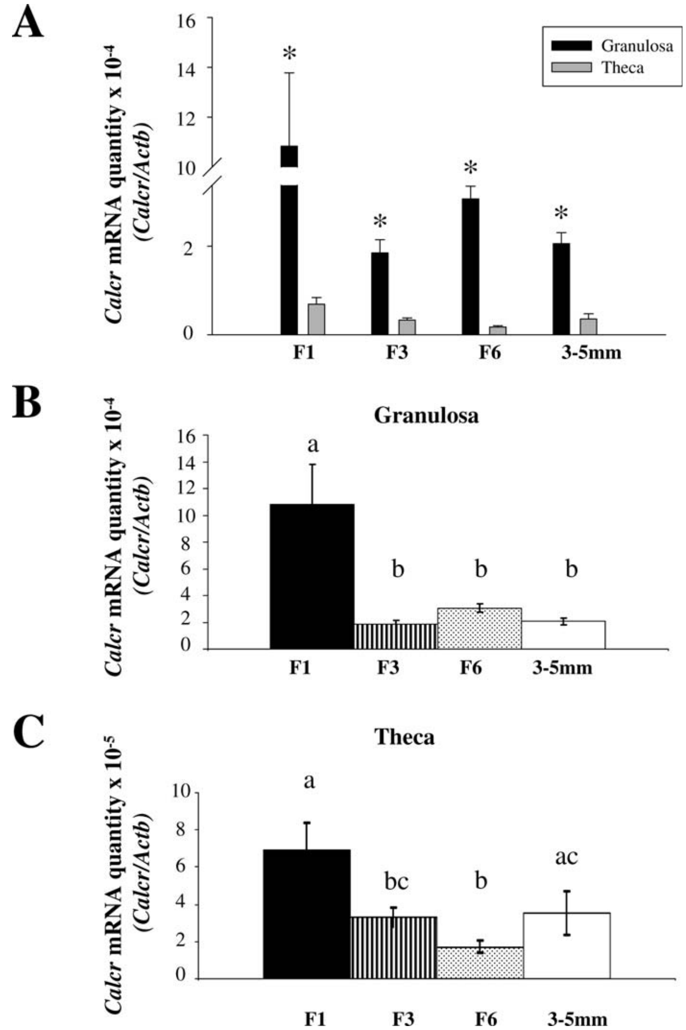

FIG. 6. **A**–**C**) *Calcr* mRNA abundance in the granulosa and theca cells of preovulatory (F1, F3, and F6) and prehierarchial (3–5 mm) follicles as measured by real-time quantitative PCR. **A**) A comparison of *Calcr* mRNA abundance between granulosa and thecal cell layers of preovulatory and prehierarchial follicles. An asterisk above each set of bars indicates significant differences at $P < 0.05$. **B**, **C**) A comparison of *Calcr* mRNA abundance within the granulosa and theca layers, respectively. Different letters above each bar indicate significant difference at $P < 0.05$. Data in **A**–**C** represent mean ± standard error of the mean (n = 5 to 6).

ovarian *Calca* mRNA revealed significant homology (>99%) with that of ultimobranchial gland as well as a predicted chicken *Calca* cDNA (GenBank accession no. XM_420997).

To determine the presence of the CALCA receptor (*Calcr*) gene in the ovarian follicles, an RT-PCR was performed using total RNA from the granulosa and thecal layers at various developmental stages (F1, F3, F6, and 3–5 mm) as well as from the kidney (as positive control). The 495-bp product corresponding to *Calcr* cDNA was amplified in the granulosa cell layers at all stages of development, as well as in the thecal cell layers from the F1 and F3 follicles (Fig. 3). A very low level of *Calcr* cDNA was amplified from the thecal cell layers of F6 and 3- to 5-mm follicles. The RNA or cDNA sample integrity was confirmed by successfully amplifying a fragment of glyceraldehyde-3-phosphate dehydrogenase cDNA for both granulosa and thecal cell layers at all developmental stages (data not shown). Furthermore, sequencing of the ovarian *Calcr* cDNA revealed significant homology (>99%) to that of kidney as well a predicted chicken *Calcr* cDNA (GenBank accession no. XM_425985).

Cellular Localization of Calca *mRNA and CALCA in the Chicken Ovary*

Calca mRNA-expressing cells were localized by in situ hybridization histochemistry in both granulosa and thecal cells of the ovary follicle (Fig. 4, A–F). No *Calca* mRNA-specific staining was observed when a sense riboprobe was used in place of antisense cRNA probe (Fig. 4, G–I). Similar to *Calca* mRNA-containing cells, CALCA-immunoreactive cells, detected by immunohistochemistry, were found distributed in both the granulosa and thecal cells (Fig. 4, J–L). Both *Calca* mRNA and CALCA were located in the cytoplasm of granulosa or thecal cells. In addition, when rabbit IgG was used in place of the anti-CALCA antibody, no CALCA-specific staining was evident in these cells (Fig. 4, M–O).

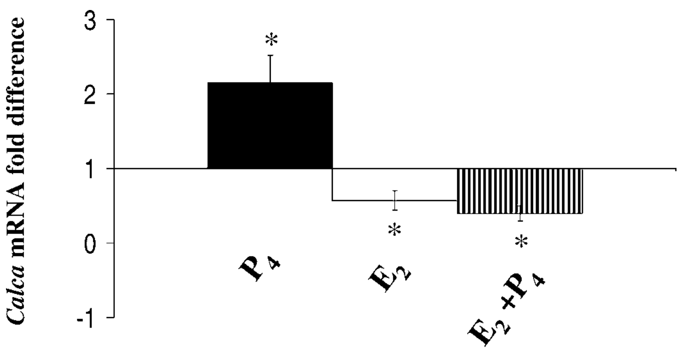

FIG. 7. Effect of P_4 and/or E_2 on *Calca* mRNA quantity in the chicken ovary. *Calca* mRNA abundance in response to E_2 and/or P_4 treatment is represented as fold difference compared with vehicle control. An asterisk above each bar indicates significant difference at $P < 0.05$ compared with vehicle control. The data represent mean ± standard error of the mean (n = 7).

Effect of Follicular Development on Calca *and* Calcr *mRNA Quantity*

The diameters of preovulatory and prehierarchial follicles (n = 6 chickens) included in this experiment are as follows: 36.2 ± 0.65 mm for F1 follicles; 27.0 ± 0.84 mm for F3 follicles; 10.5 ± 0.43 mm for F6 follicles; and 3–5 mm for prehierarchial follicles. The granulosa cell layers of all preovulatory follicles (F1, F3, and F6) and prehierarchial follicles (3–5 mm) contained significantly greater abundance of *Calca* mRNA versus the thecal cell layer ($P < 0.05$; Fig. 5A). Within the granulosa cell layer, the quantity of *Calca* mRNA in F1 follicles was significantly higher ($P < 0.05$; Fig. 5B) compared with that in the granulosa cell layer of other follicles studied (F3, F6, and 3–5 mm). Relative to F3 and F6 follicles, the granulosa cell layers of the prehierarchial follicle (3–5 mm) contained significantly higher *Calca* mRNA quantity ($P < 0.05$). In the thecal cell layer, *Calca* mRNA quantity was not significantly different among preovulatory or prehierarchial follicles ($P > 0.05$; Fig. 5C). Melting curve analyses showed the presence of a single PCR product for *Calca* mRNA or *Actb* mRNA, confirming the specificity of the reaction (data not shown).

In addition, the granulosa cell layers also contained significantly greater levels of *Calcr* mRNA versus the thecal cell layers ($P < 0.05$; Fig. 6A) for both preovulatory and prehierarchial follicles. Furthermore, the granulosa layer of the F1 follicle contained significantly higher *Calcr* mRNA quantity compared with that in the granulosa layer of all other follicles ($P < 0.05$; Fig. 6B). Within the thecal cell layer, *Calcr* mRNA quantity was significantly higher in the F1 follicle as well as in the prehierarchial follicles (3–5 mm) relative to F3 and F6 follicles ($P < 0.05$; Fig. 6C). Melting curve analyses showed the presence of a single PCR product for *Calcr* mRNA or *Actb* mRNA, confirming the specificity of the reaction (data not shown).

Effect of Ovarian Steroids on Ovarian Calca *mRNA Quantity*

E_2 and/or P_4 treatment for 7 days resulted in a significant increase in oviduct weights (data not shown). Ovarian *Calca* mRNA quantity was significantly elevated (2.2-fold higher) in response to P_4 treatment ($P < 0.05$; Fig. 7) versus vehicle treatment. E_2 or a combination of E_2 and P_4, however, caused a significant decrease (0.4- or 0.5-fold, respectively) in ovarian *Calca* mRNA abundance relative to vehicle treatment ($P < 0.05$, Fig. 7). Melting curve analyses showed the presence of a single PCR product for *Calca* mRNA or *Actb* mRNA, confirming the specificity of the reactions (data not shown).

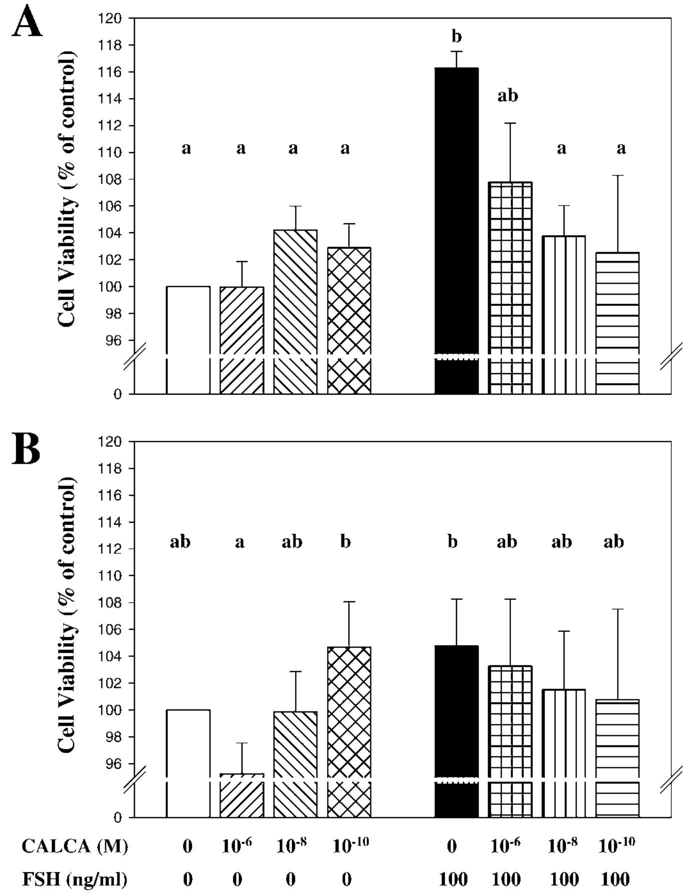

FIG. 8. Effect of chicken CALCA and/or FSH on granulosa cell viability. Granulosa cells were dispersed from the 6- to 8-mm follicles of two hens, pooled, and treated in triplicate with or without FSH (100 ng/ml) and chicken CALCA (0, 10^{-10}, 10^{-8}, 10^{-6} M) for 12 h (**A**) and 24 h (**B**). Cell viability was measured using the CellTiter-Blue assay, and viability is expressed as a percentage of vehicle control ± SEM (n = 4 replicates of two animals each). Different letters above each bar indicate significant difference at $P < 0.05$.

Effect of Chicken CALCA and/or FSH on Granulosa Cell Viability

To determine the role of chicken CALCA on granulosa cell viability in preheirarchial follicles (6–8 mm), granulosa cells were cultured for 12 and 24 h with various concentrations of chicken CALCA and/or FSH, with viability determined by the CellTiter-Blue assay. At 12 h, FSH treatment significantly increased cell viability compared with vehicle treatments ($P < 0.05$; Fig. 8A), indicating that the cells were responsive to FSH treatment. This stimulation was significantly inhibited by a combination of FSH and CALCA at 10^{-8} and 10^{-10} M ($P < 0.05$; Fig. 8A). However, no significant changes in cell viability were observed at 12 h in response to CALCA treatment alone. There was no difference in cell viability in response to either CALCA or a combination of FSH and CALCA following 24 h of incubation (Fig. 8B).

DISCUSSION

The present study is the first to characterize the expression of *Calca* in the ovary of any vertebrate species. By RT-PCR, we identified *Calca* mRNA in the chicken adipose, liver, hypothalamus, kidney, spleen, and ovary. We found that the nucleotide sequence of ovarian *Calca* cDNA was homologous (>99% identities) to the ultimobranchial gland-derived *Calca* [25], indicating that ovarian- and ultimobranchial gland-derived CALCA may share biologic properties. We confirmed the expression of *Calca* in the chicken ovary by in situ hybridization and immunohistochemistry. Both *Calca* mRNA and CALCA were localized in the granulosa and theca cell layers within the chicken ovarian follicles at various maturation stages. A recent study by Hidaka et al. [26] hints at the presence of *Calca* mRNA in the ovary, in which *Calca* cDNA was amplified as a faint band from trout and rat ovarian RNA by RT-PCR. However, in the same study, the presence of *Calca* mRNA in the ovary could not be confirmed by in situ hybridization. Nevertheless, *Calca* mRNA has been documented in the testis of rainbow trout [26], as well as endometrium of rats [14], baboons [15], and humans [16], suggesting a role for CALCA in reproduction.

In the present study, *Calcr* gene expression was identified in both the granulosa and theca cell layers of follicles at various maturation stages. Previous studies using receptor binding assays have demonstrated CALCA binding sites in the shell gland endometrium of chicken [27] and guinea fowl [28], as well as in rat Leydig cells [29, 30], suggesting the presence of a specific plasma membrane receptor for CALCA within these reproductive tissues. Moreover, calcitonin receptor-like molecules have also been found in the testis and ovary of flounder [31]. Taken together, these findings further support a possible role for CALCA and its receptor in both male and female reproduction.

While there are no other reports available on the possible functions of ovarian CALCA or related peptides, earlier studies have found a temporal relationship between circulating CALCA levels and ovarian development. In trout and eel, circulating CALCA levels were found to fluctuate with respect to ovarian activity, with a peak observed at the time of ovulation [11, 12]. Plasma CALCA levels were also elevated in the Japanese quail at the time preceding eggshell deposition. However, plasma CALCA levels remained constant throughout the ovulatory cycle in egg-laying hens [32]. In addition, removal of the ultimobranchial gland, the primary source of CALCA in chickens, did not appear to affect egg production [33]. It is possible that the ovarian CALCA found in the present study may possibly compensate for the ultimobranchial gland-derived CALCA at the ovarian level.

In the present study, we found that follicular maturation influenced both *Calca* and *Calcr* gene expression. The F1 preovulatory follicle granulosa layer showed a dramatic and abrupt increase in *Calca* mRNA abundance compared with that of any other preovulatory follicle. Similarly, the granulosa layer of the F1 follicle also had the greatest level of *Calcr* gene expression, suggesting an important role of CALCA signaling in the most mature preovulatory follicle destined for ovulation. In contrast, relatively low levels of both *Calca* mRNA and *Calcr* mRNA were found in the smaller preovulatory and prehierarchial follicle granulosa cells. Since the granulosa cells of the prehierarchial follicles remain undifferentiated compared with the fully mature granulosa cells found in the F1 follicle [34], ovarian CALCA may function as a local regulator of cell differentiation and therefore influence follicular maturation. Likewise, a closely related peptide belonging to *Calca* gene family, adrenomedullin, has previously been identified in rat granulosa cells, where it is believed to function as an autocrine regulator of cellular differentiation [35].

To further investigate the role of CALCA on cell differentiation, we treated cultured granulosa cells obtained from prehierarchial follicles with chicken CALCA and/or FSH, and we quantified cell viability using a fluorescence-based detection method. Despite previous reports of CALCA altering proliferation in various cell types (i.e., lactotrophes [36], mammary epithelial cells [37], prostate cancer cell lines [38], HEK cell line [39]), the present study failed to find any significant changes in granulosa cell viability when CALCA was applied alone. However, a combination of CALCA and FSH did cause a significant decrease in cellular viability compared with cells treated with FSH alone following 12 h of treatment. This decreased viability may represent the ability of CALCA to inhibit FSH-induced proliferation [40], as previous reports have correlated cell viability with mitochondrial activity [41] and cell proliferation [42, 43]. Alternatively, CALCA may have inhibited other FSH-induced functions in granulosa cells, such as promoting cell survival, increasing steroidogenic potential, or inhibiting apoptosis [44–46]. Following 24 h of CALCA treatment, however, this inhibitory effect was not observed, possibly due to degradation of CALCA in culture media over time.

Based on CALCA's ability to decrease viability of FSH-stimulated granulosa cells, we propose that intraovarian CALCA may be functioning as a local regulator of granulosa cell proliferation in the preheirarchial follicles, thereby prohibiting the entry of numerous follicles into the preovulatory hierarchy. In the viability assay, the greatest inhibition was observed with the lowest concentration of CALCA (10^{-10} M), whereas the highest CALCA concentration (10^{-6} M) did not further exaggerate this response, possibly indicating receptor saturation at such supraphysiologic levels. Interestingly, the preheirarchial follicles were also found to have the lowest level of *Calca* mRNA expression and contained the greatest amount of FSH receptors [47, 48], and therefore may be the most susceptible to this inhibitory effect. In addition, these prehierarchial follicles are the main producers of estradiol [49], which was shown to decrease total ovarian *Calca* mRNA expression (Fig. 7). In this manner, estradiol may be acting to maintain the low CALCA concentrations within the prehierarchial follicles so as to enable the granulosa cells to be maximally responsive to the inhibitory effect of CALCA.

The role for CALCA in follicular maturation is further supported by the ability of gonadal steroids, which fluctuate throughout the ovulatory cycle, to alter ovarian *Calca* expression. In the present study, treatment of sexually immature chickens with estradiol caused a significant reduction in ovarian *Calca* mRNA levels. Previous studies have shown that estradiol inhibits *Calca* expression in the endometrium [14] and pituitary [50]. In contrast, estradiol administration to goldfish stimulated ultimobranchial CALCA secretion [51], and it had similar effects in vitro on CALCA secretion from rat thyroid glands [52]. This variable response may further substantiate the various roles for CALCA in different organs. We also found that P_4 administration to sexually immature chickens caused a significant increase in *Calca* mRNA levels in the ovary compared with vehicle treatment. As the follicle matures in the chicken ovary, the granulosa cell layer undergoes differentiation to produce large quantities of P_4 [53]. The largest, most mature follicle (F1), which produces the highest amount of P_4, was found in this study to express the highest levels of *Calca* mRNA. In support of our data, P_4 treatment has been shown to increase *Calca* gene expression in rat thyroid glands [52] and in the endometrium of humans and baboons [6, 15, 16].

Whereas P_4 administration influences *Calca* mRNA expression in the chicken ovary, CALCA treatment has been shown to significantly decrease basal and hCG-induced P_4 secretion from rat granulosa cells in vitro in a post-cAMP-dependent pathway [54]. However, we did not observe any change in P_4 secretion from the granulosa cells of F1 follicles cultured for 2 h with chicken CALCA at 10^{-6}, 10^{-8}, and 10^{-10} M $\pm$ 5 ng/ml oLH (data not shown). Such a lack of response to CALCA treatment may be due to differences in the source of ovarian P_4 production between mammalian and avian species. Alternatively, the granulosa cells from F1 follicles are possibly resistant to the inhibitory effect of CALCA on P_4 production in the chicken. Although not immediately involved in steroidogenesis regulation, the large increase in both *Calca* and *Calcr* mRNA observed in the granulosa layer of the F1 follicle may facilitate ovulation through increased activation of plasminogen activators. As the follicle matures, the activity of plasminogen activator, a protease believed to be involved in follicular rupture and ovulation, drastically increases in both the granulosa and theca cell layers [55]. Interestingly, CALCA has been shown to increase plasminogen activator production up to 1000-fold in porcine renal tubular cells in vitro [56]. It is therefore possible that increased CALCA within the F1 follicle increases plasminogen activator, thereby aiding in follicular rupture and ovulation.

In conclusion, we found that *Calca* mRNA, CALCA, and *Calcar* mRNA are expressed by the granulosa and thecal cells in the chicken ovarian follicles. A dramatic increase in both *Calca* mRNA and *Calcr* mRNA abundances in the F1 follicle granulosa cell layer suggests its possible role in follicular maturation and granulosa cell differentiation. In addition, both E_2 and P_4 were found to influence *Calca* mRNA abundance in the chicken ovary. Furthermore, we found that chicken CALCA significantly decreased the FSH-stimulated viability of prehierarchial follicular granulosa cells. Further studies are therefore required to characterize additional role(s) of CALCA and CALCR in the ovary.

REFERENCES

1. Chan AS, Cipera JD, Belanger LF. The ultimobranchial gland of the chick and its response to a high calcium diet. Rev Can Biol 1969; 28:19–31.
2. Anderson RE, Schraer H, Gay CV. Ultrastructural immunocytochemical localization of carbonic anhydrase in normal and calcitonin-treated chick osteoclasts. Anat Rec 1982; 204:9–20.
3. Sommerville BA, Fox J. Changes in renal function of the chicken associated with calcitonin and parathyroid hormone. Gen Comp Endocrinol 1987; 66:381–386.
4. Russwurm S, Stonans I, Stonane E, Wiederhold M, Luber A, Zipfel PF, Deigner HP, Reinhart K. Procalcitonin and CGRP-1 mrna expression in various human tissues. Shock 2001; 16:109–112.
5. Sjoberg HE, Arver S, Bucht E. High concentration of immunoreactive calcitonin of prostatic origin in human semen. Acta Physiol Scand 1980; 110:101–102.
6. Ding YQ, Zhu LJ, Bagchi MK, Bagchi IC. Progesterone stimulates calcitonin gene expression in the uterus during implantation. Endocrinology 1994; 135:2265–2274.
7. Ren Y, Chien J, Sun YP, Shah GV. Calcitonin is expressed in gonadotropes of the anterior pituitary gland: its possible role in paracrine regulation of lactotrope function. J Endocrinol 2001; 171:217–228.
8. Bucht E, Telenius-Berg M, Lundell G, Sjoberg HE. Immunoextracted calcitonin in milk and plasma from totally thyroidectomized women. Evidence of monomeric calcitonin in plasma during pregnancy and lactation. Acta Endocrinol (Copenh) 1986; 113:529–535.
9. Tverberg LA, Gustafson MF, Scott TL, Arzumanova IV, Provost ER, Yan AW, Rawie SA. Induction of calcitonin and calcitonin receptor expression in rat mammary tissue during pregnancy. Endocrinology 2000; 141:3696–3702.
10. Shah GV, Epand RM, Orlowski RC. Calcitonin inhibition of prolactin secretion in isolated rat pituitary cells. J Endocrinol 1988; 116:279–286.
11. Bjornsson BT, Haux C, Forlin L, Deftos LJ. The involvement of calcitonin in the reproductive physiology of the rainbow trout. J Endocrinol 1986; 108:17–23.
12. Yamauchi H, Orimo H, Yamauchi K, Takano K, Takahashi H. Increased calcitonin levels during ovarian development in the eel, Anguilla japonica. Gen Comp Endocrinol 1978; 36:526–529.
13. Cressent M, Elie C, Taboulet J, Moukhtar MS, Milhaud G. Calcium regulating hormones during the estrous cycle of the rat. Proc Soc Exp Biol Med 1983; 172:158–162.
14. Zhu LJ, Cullinan-Bove K, Polihronis M, Bagchi MK, Bagchi IC. Calcitonin is a progesterone-regulated marker that forecasts the receptive state of endometrium during implantation. Endocrinology 1998; 139: 3923–3934.
15. Kumar S, Brudney A, Cheon YP, Fazleabas AT, Bagchi IC. Progesterone induces calcitonin expression in the baboon endometrium within the window of uterine receptivity. Biol Reprod 2003; 68:1318–1323.
16. Kumar S, Zhu LJ, Polihronis M, Cameron ST, Baird DT, Schatz F, Dua A, Ying YK, Bagchi MK, Bagchi IC. Progesterone induces calcitonin gene expression in human endometrium within the putative window of implantation. J Clin Endocrinol Metab 1998; 83:4443–4450.
17. Zhu LJ, Bagchi MK, Bagchi IC. Attenuation of calcitonin gene expression in pregnant rat uterus leads to a block in embryonic implantation. Endocrinology 1998; 139:330–339.
18. Gilbert AB, Evans AJ, Perry MM, Davidson MH. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (Gallus domesticus). J Reprod Fertil 1977; 50:179–181.
19. Di Nino DL, Linsenmayer TF. Positive regulation of endochondral cartilage growth by perichondrial and periosteal calcitonin. Endocrinology 2003; 144:1979–1983.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402–408.
21. Dunn IC, Lewis PD, Wilson PW, Sharp PJ. Acceleration of maturation of FSH and LH responses to photostimulation in prepubertal domestic hens by oestrogen. Reproduction 2003; 126:217–225.
22. Liu HK, Bacon WL. Changes in egg production rate induced by progesterone injection in broiler breeder hens. Poult Sci 2005; 84:321–327.
23. Tilly JL, Johnson AL. Presence and hormonal control of plasminogen activator in granulosa cells of the domestic hen. Biol Reprod 1987; 37: 1156–1164.
24. Maddineni SR, Krzysik-Walker SM, Ocon-Grove OM, Motch SM, Hendricks GL III, Ramachandran R. Calcitonin is expressed in the chicken pituitary gland: influence of gonadal steroids and sexual maturation. Cell Tissue Res 2007; 327:521–528.
25. Lasmoles F, Jullienne A, Day F, Minvielle S, Milhaud G, Moukhtar MS. Elucidation of the nucleotide sequence of chicken calcitonin mRNA: direct evidence for the expression of a lower vertebrate calcitonin-like gene in man and rat. EMBO J 1985; 4:2603–2607.
26. Hidaka Y, Suzuki M. Identification and tissue distribution of mRNAs encoding salmon-type calcitonins-IV and -V in the rainbow trout. J Mol Endocrinol 2004; 32:963–974.
27. Ieda T, Takahashi T, Saito N, Yasuoka T, Kawashima M, Izumi T, Shimada K. Changes in calcitonin receptor binding in the shell gland of laying hens (Gallus domesticus) during the oviposition cycle. J Poult Sci 2001; 38:203–212.
28. Ogawa H, Takahashi T, Kuwayama T, Kawashima M. Presence of calcitonin receptors in shell gland of the guineafowl and changes in binding property during an oviposition cycle. Poult Sci 2003; 82:1302–1306.
29. Chausmer AB, Stevens MD, Severn C. Autoradiographic evidence for a calcitonin receptor on testicular Leydig cells. Science 1982; 216:735–736.
30. Chausmer A, Stuart C, Stevens M. Identification of testicular cell plasma membrane receptors for calcitonin. J Lab Clin Med 1980; 96:933–938.
31. Suzuki N, Suzuki T, Kurokawa T. Cloning of a calcitonin gene-related peptide receptor and a novel calcitonin receptor-like receptor from the gill of flounder, Paralichthys olivaceus. Gene 2000; 244:81–88.
32. Dacke CG, Boelkins JN, Smith WK, Kenny AD. Plasma calcitonin levels in birds during the ovulation cycle. J Endocrinol 1972; 54:369–370.
33. Speers GM, Perey DYE, Brown DM. Effect of ultimobranchialectomy in the laying hen. Endocrinology 1970; 87:1292–1297.
34. Tilly JL, Kowalski KI, Johnson AL. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod 1991; 44:305–314.

35. Abe K, Minegishi T, Tano M, Hirakawa T, Tsuchiya M, Kangawa K, Kojima M, Ibuki Y. Expression and effect of adrenomedullin on rat granulosa cell. Endocrinology 1998; 139:5263–5266.
36. Shah GV, Chien J, Sun YP, Puri S, Ravindra R. Calcitonin inhibits anterior pituitary cell proliferation in the adult female rats. Endocrinology 1999; 140:4281–4291.
37. Ishii A, Nakamura M, Nakamura A, Takeda K, Han B, Kakudo K. Expression of calcitonin receptor in rat mammary gland during lactation. Endocr J 2006; 53:317–324.
38. Chien J, Ren Y, Qing Wang Y, Bordelon W, Thompson E, Davis R, Rayford W, Shah G. Calcitonin is a prostate epithelium-derived growth stimulatory peptide. Mol Cell Endocrinol 2001; 181:69–79.
39. Findlay DM, Raggatt LJ, Bouralexis S, Hay S, Atkins GJ, Evdokiou A. Calcitonin decreases the adherence and survival of HEK-293 cells by a caspase-independent mechanism. J Endocrinol 2002; 175:715–725.
40. Davis AJ, Brooks CF, Johnson PA. Follicle-stimulating hormone regulation of inhibin alpha- and beta(B)-subunit and follistatin messenger ribonucleic acid in cultured avian granulosa cells. Biol Reprod 2001; 64: 100–106.
41. Springer JE, Azbill RD, Carlson SL. A rapid and sensitive assay for measuring mitochondrial metabolic activity in isolated neural tissue. Brain Res Brain Res Protoc 1998; 2:259–263.
42. Nakayama GR, Caton MC, Nova MP, Parandoosh Z. Assessment of the Alamar Blue assay for cellular growth and viability in vitro. J Immunol Methods 1997; 204:205–208.
43. Bounous DI, Campagnoli RP, Brown J. Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assays using chicken splenocytes. Avian Dis 1992; 36:1022–1027.
44. Johnson AL, Bridgham JT. Regulation of steroidogenic acute regulatory protein and luteinizing hormone receptor messenger ribonucleic acid in hen granulosa cells. Endocrinology 2001; 142:3116–3124.
45. Hernandez AG, Bahr JM. Role of FSH and epidermal growth factor (EGF) in the initiation of steroidogenesis in granulosa cells associated with follicular selection in chicken ovaries. Reproduction 2003; 125:683–691.
46. Johnson AL, Bridgham JT, Witty JP, Tilly JL. Susceptibility of avian ovarian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of bcl-xlong gene expression. Endocrinology 1996; 137:2059–2066.
47. You S, Bridgham JT, Foster DN, Johnson AL. Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. Biol Reprod 1996; 55:1055–1062.
48. Ritzhaupt LK, Bahr JM. A decrease in FSH receptors of granulosa cells during follicular maturation in the domestic hen. J Endocrinol 1987; 115: 303–310.
49. Robinson FE, Etches RJ. Ovarian steroidogenesis during follicular maturation in the domestic fowl (Gallus domesticus). Biol Reprod 1986; 35:1096–1105.
50. Sun YP, Lee TJ, Shah GV. Calcitonin expression in rat anterior pituitary gland is regulated by ovarian steroid hormones. Endocrinology 2002; 143: 4056–4064.
51. Suzuki N, Yamamoto K, Sasayama Y, Suzuki T, Kurokawa T, Kambegawa A, Srivastav AK, Hayashi S, Kikuyama S. Possible direct induction by estrogen of calcitonin secretion from ultimobranchial cells in the goldfish. Gen Comp Endocrinol 2004; 138:121–127.
52. Greenberg C, Kukreja SC, Bowser EN, Hargis GK, Henderson WJ, Williams GA. Effects of estradiol and progesterone on calcitonin secretion. Endocrinology 1986; 118:2594–2598.
53. Porter TE, Hargis BM, Silsby JL, el Halawani ME. Differential steroid production between theca interna and theca externa cells: a three-cell model for follicular steroidogenesis in avian species. Endocrinology 1989; 125:109–116.
54. Tsai SC, Lu CC, Chen JJ, Chiao YC, Wang SW, Hwang JJ, Wang PS. Inhibition of salmon calcitonin on secretion of progesterone and GnRH-stimulated pituitary luteinizing hormone. Am J Physiol 1999; 277:E49–E55.
55. Politis I, Wang L, Turner JD, Tsang BK. Changes in tissue-type plasminogen activator-like and plasminogen activator inhibitor activities in granulosa and theca layers during ovarian follicle development in the domestic hen. Biol Reprod 1990; 42:747–754.
56. Dayer JM, Vassalli JD, Bobbitt JL, Hull RN, Reich E, Krane SM. Calcitonin stimulates plasminogen activator in porcine renal tubular cells: LLC-PK1. J Cell Biol 1981; 91:195–200.