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Expression of Gap Junctional Proteins Connexin 43, 32, and 26 Throughout Follicular Development and Atresia in Cows

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Detection of connexin (Cx) proteins has been used as an indicator of the presence of structural and functional gap junctions in tissues. To examine the role of gap junctions during follicular growth and atresia, the presence of three major connexins, Cx43, Cx32, and Cx26, was evaluated in bovine ovaries by using immunohistochemistry and Western immunoblot analysis. Cx43 was not present in primordial follicles, but was present in granulosa cells of primary/secondary and antral follicles. Cx43 also was present on the borders between granulosa cells and the oocyte. Expression of Cx43 increased in healthy developing antral follicles, but decreased during follicular atresia. Cx32 was not present in healthy follicles but was present in granulosa cells of atretic antral, and especially small antral follicles. Cx26 was present in the oocyte of primordial and primary/secondary follicles, and in the granulosa and/or thecal cell layers of healthy antral follicles. The percentage of healthy antral follicles that expressed Cx26 also increased during follicular development, but decreased during atresia. Cx32 and Cx26 also were detected in ovarian blood vessels and in stromal tissues adjacent to the tunica albuginea in some ovaries. The pattern of expression of these Cx indicates that gap junctional proteins may be involved in the control of follicular growth and atresia in cows.

Key Words: Bovine; ovaries; gap junctions; immunohistochemistry; Western analysis; cell–cell interactions.

Introduction

During growth and development, ovarian follicles are maintained as healthy, growing structural units despite a high rate of cell turnover *(1)*. When follicles undergo atresia, all cells within one follicle are removed in an apoptotic manner *(1)*. This makes ovarian follicles ideal models for studying how cell function is coordinated during growth or regression of healthy tissue. Cell–cell communication through gap junctional channels, formed by specific proteins known as connexins (Cx), is an important mechanism for regulating cell growth and regression *(2–8)*.

Connexins comprise a family of related proteins that may combine heterotypically or homotypically to form gap junctions *(9,10)*. Generally, Cx are classified on the basis of the predicted or actual (determined by Western analysis) size of their proteins *(3,10)*. Expression of different Cx or combinations of Cx changes gap junctional channel characteristics and this may be one way gap junctional communication is regulated *(5,10–12)*. Additionally, each Cx appears to be expressed in a cell-specific manner that may change in response to hormone or growth factor stimulation *(13)*.

Gap junctions and Cx are present in ovarian follicles of several species, and they may play a role in control of oocyte maturation and follicular development *(7,14–20)*. Cell–cell coupling of the oocyte with cumulus cells maintains the meiotic arrest of the oocyte until maturation; uncoupling of gap junctions at the proper time leads to ovulation *(14,15,21)*. An in vitro model of rat follicle development has shown that the oocyte directs follicle morphogenesis through a gap junction-mediated mechanism *(20)*. The pattern of expression of gap junctional proteins for Cx43, Cx32, and Cx26 in sheep ovaries across all stages of follicular development suggests that these gap junctional proteins may be important in the regulation of follicular growth and atresia in sheep *(19)*. Cx26 is present in the oocytes of primordial and primary follicles of sheep and Cx32 is in mature mouse and bovine oocytes prior to ovulation *(7)*. The preovulatory LH surge may cause the numbers of gap junctions to decrease in preovulatory follicles of rats *(7)*. The expression of Cx43 and Cx26 or the number and size of gap junctions increases during follicular development in many species *(7)*. During atresia, expression of Cx43 in granulosa layers appears to decrease or be detected mostly in thecal layers of rats and sheep. However, it has yet to be determined whether changes in gap junction expression are associated with follicular growth and/or regression of fol-

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licles. Therefore, in this study, we examined the patterns of expression of Cx43, Cx32, and Cx26 during follicular growth and atresia in cows. In this article, we present new data demonstrating that Cx32 protein was only present in atretic follicles in cows and, in addition, that patterns of expression of Cx43 and Cx26 suggest that these connexins are involved in the maintenance of healthy follicles.

Results

Experiment 1

Serial sections of bovine ovaries were used for evaluation of Cx43, Cx32, and Cx26 proteins in ovarian tissues so that the pattern of staining for each connexin could be evaluated and compared across the same follicles within each ovary. In all antral follicles, the staining for Cx43 and Cx26 was punctate, but Cx32 staining of the granulosa layer of atretic follicles was cytoplasmic and diffuse (Figs. 1–3). None of the controls (normal rabbit serum and preabsorbed antibody) showed positive staining (data not shown).

Cx43 protein was not seen in primordial follicles (n > 130), but in preantral primary and secondary follicles (n = 101) punctate staining for Cx43 was detected in the granulosa layer of 95% of the follicles (Fig. 1A). All healthy small (n = 40) and medium (n = 9) antral follicles contained Cx43 in the granulosa layer and the staining was predominantly punctate with some diffuse cytoplasmic staining (Fig. 1C). For all healthy large (n = 13) antral follicles, Cx43 was present in granulosa cells, and 15% of these follicles also had weak staining in theca. Cx43 was also detected at the border between granulosa cells and the oocyte in antral follicles (Figs. 1A,B). For atretic small (n = 20) antral follicles, 6% had staining for Cx43 in granulosa and/or thecal layers. For atretic medium (n = 11) and large (n = 9) antral follicles, Cx43 was detected in the granulosa cells of 18% and 22% of the follicles, respectively. The results of Cx43 immunohistochemistry are summarized in Fig. 4A,B.

Cx32 protein was not detected in any primordial, preantral, or healthy antral follicles. In contrast, for all atretic small antral follicles Cx32 was present in the cytoplasm of the granulosa cells (Fig. 2A). Cx32 also was present in the cytoplasm of the granulosa cells of 45% of medium atretic follicles and 11% of large atretic follicles and was occasionally detected in the cumulus cells (Fig 2B). The results of Cx32 immunohistochemistry are summarized in Fig. 4C,D.

Cx26 protein was present in the oocyte of all primordial follicles (Fig. 3A). For preantral follicles, Cx26 was present in either the oocyte (91%) and/or the granulosa cells (11%, Fig. 3A). Cx26 also was detected in the granulosa and/or theca of 27% of small, 33% of medium, and all large healthy antral follicles (Fig. 3B). For atretic antral follicles, Cx26 was detected in the granulosa and/or theca of 35% of small, 55% of medium, and 33% of large follicles. The results of Cx26 immunohistochemistry are summarized in Fig. 4E,F.

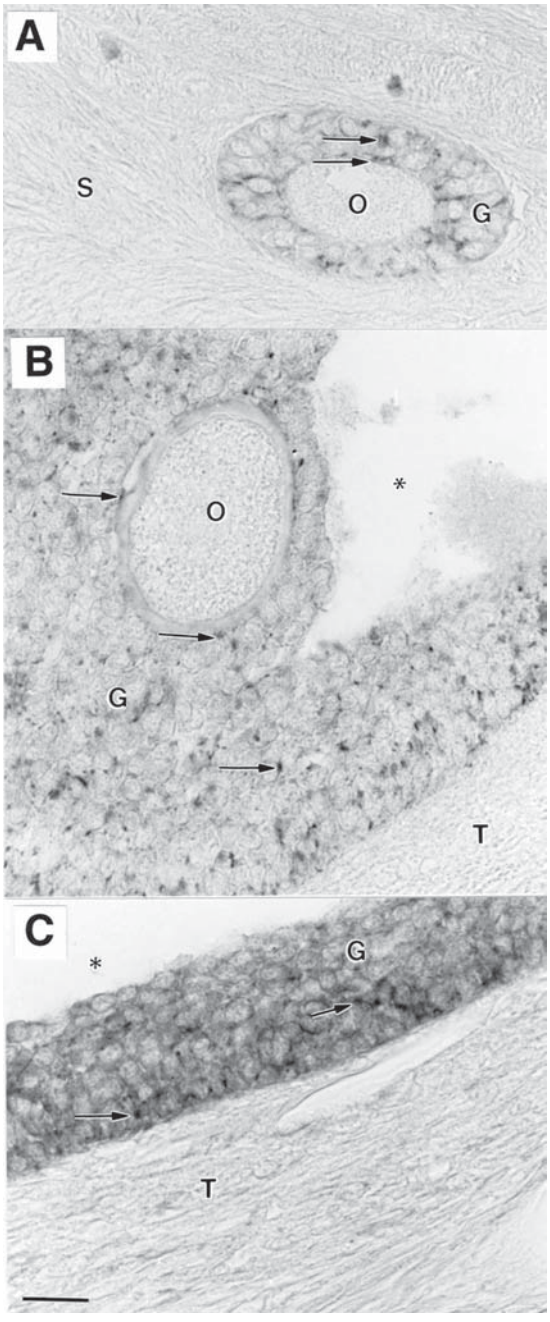

Fig. 1. Immunohistochemical localization of Cx43 in **(A)** a secondary follicle and oocyte (O), **(B)** an early antral follicle and oocyte, and **(C)** the granulosa (G) and thecal (T) layers of a healthy small antral follicle. Note punctate staining (arrows) in granulosa but not thecal layers, (A–C)**,** and at the cumulous-oocyte borders, (A-B). Sizes of follicles were determined as described in Methods. Control sections did not show positive staining (not shown because they were similar to unstained sections of the follicles shown above). S, stroma; *, antrum. Bar, 20 μm.

Additionally, in most ovaries, Cx32 and Cx26 were detected in ovarian blood vessels (data not shown). In some ovaries, Cx32 (Fig. 2C) and Cx26 (Fig. 3C) were detected in the surface epithelium (tunica albuginea) of the ovary

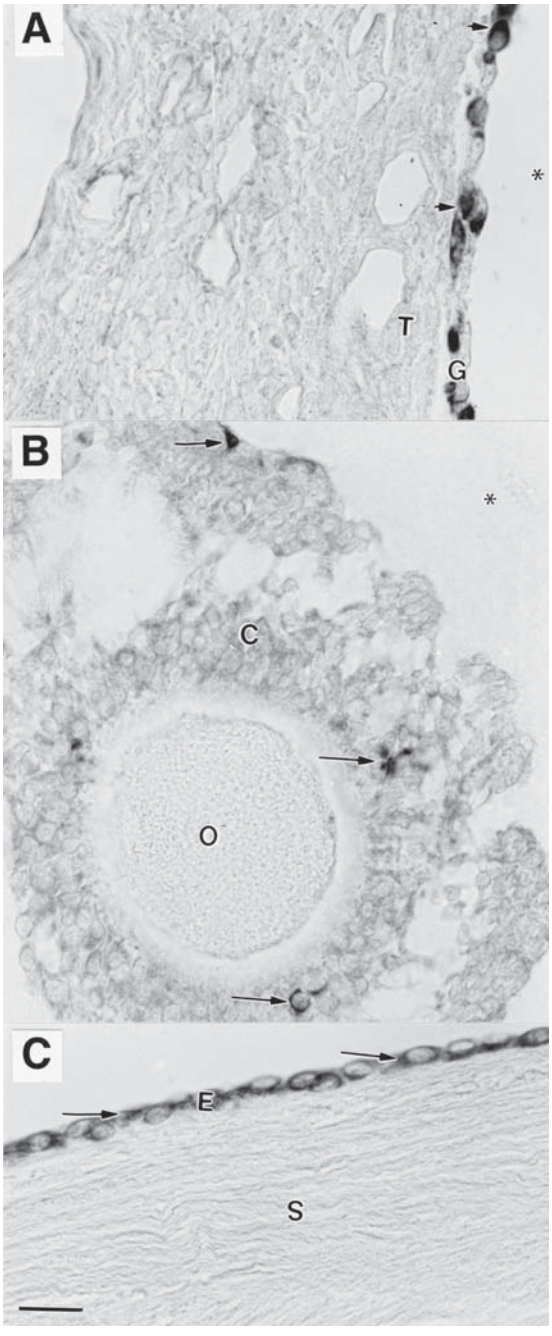

Fig. 2. Immunohistochemical localization of Cx32 in **(A)** granulosa (G) and thecal (T) layers of an atretic small antral follicle, **(B)** the cumulus layer (C) of an oocyte (O) from an atretic small antral follicle, and **(C)** the surface epithelium (E) of the ovary. Note cytoplasmic staining (arrowheads) of granulosa, (A–B), but not thecal layers, (B), and positive staining (arrows) of epithelial cells, (C). Sizes of follicles were determined as described in Methods. Control sections did not show positive staining (not shown because they were similar to unstained sections of the follicles shown above). S, stroma; *, antrum. Bar, 20 μm.

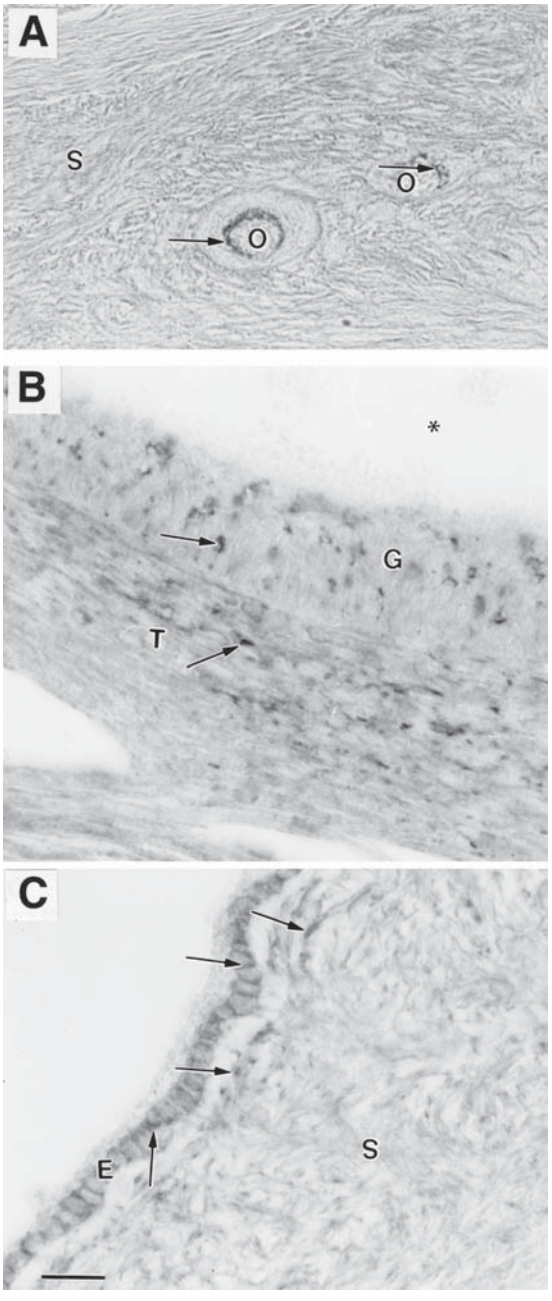

Fig. 3. Immunohistochemical localization of Cx26 in **(A)** primordial and secondary follicles, **(B)** granulosa (G) and thecal (T) layers of a healthy small antral follicle, and **(C)** surface epithelium (E) and stromal (S) areas of the ovary. Note positive staining of oocytes (O; arrows) in the primordial and secondary follicles, (A), punctate staining (arrows) in granulosa and thecal layers of antral follicles, (B), and positive staining of epithelial cells of the ovary, (C). Sizes of follicles were determined as described in Materials and Methods. Control sections did not show positive staining (not shown because they were similar to unstained sections of the follicles shown above). S, stroma; *, antrum. Bar, 20 μm.

and there was heterogenous staining for these connexins in the stromal areas of the ovary (Fig. 3C).

Experiment 2

A representative autoradiograph of follicular granulosa and thecal layers evaluated for Cx43 by Western analysis is shown in Fig. 5A. Specificity of the Cx43 antibody was

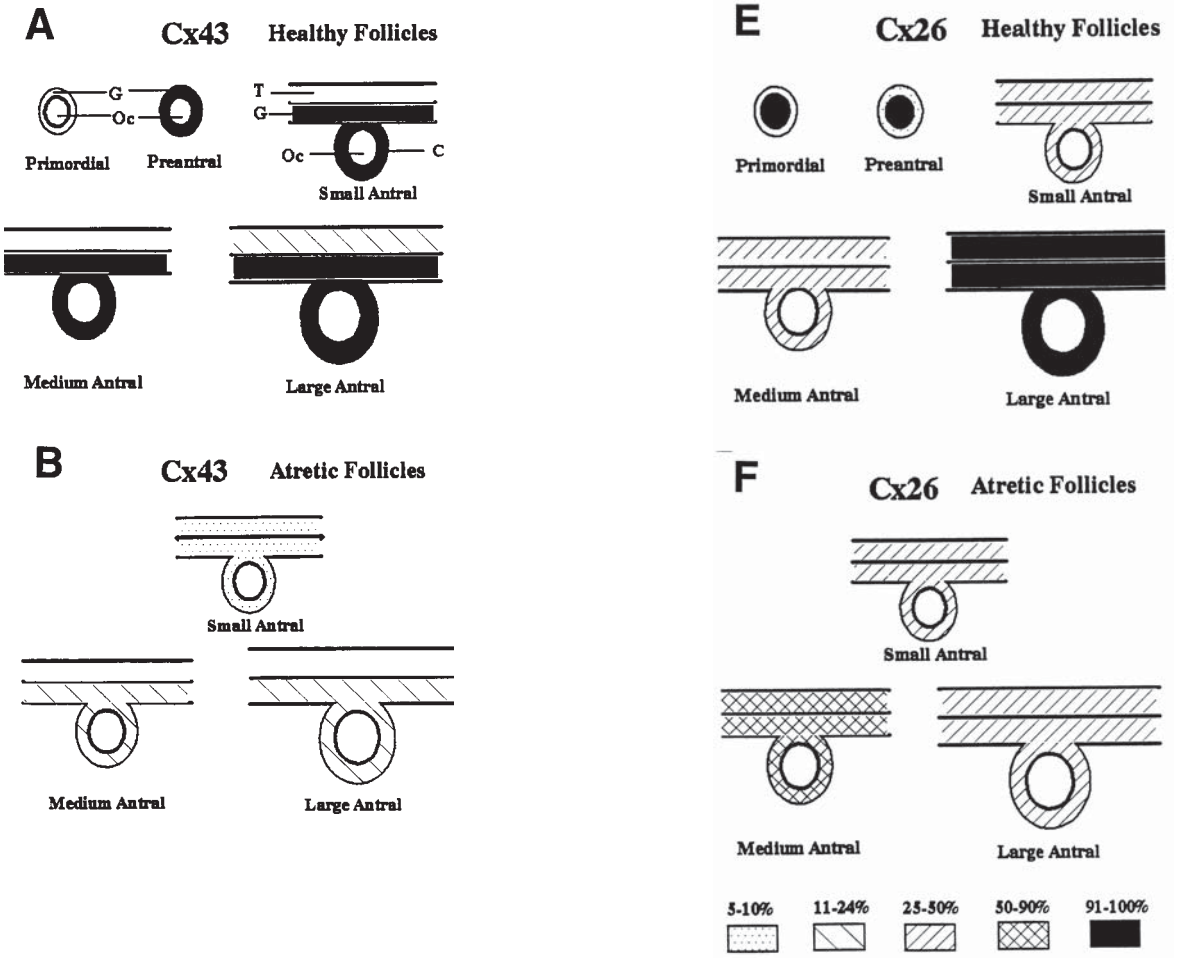

Fig. 4. Staining patterns for Cx43, **(A,B)**, Cx32, **(C,D)**, and Cx26, **(E,F)**, across all stages of follicular growth (A, C, and E) or atresia (B, D, and F). As shown in the legend, fills indicate the percentage of follicles with positive staining in that particular region of the follicle. The solid fill (black bar) shows that over 91% of follicles were stained and unfilled shows no staining in that region of the follicle. Sizes of follicles were determined as described in Methods. See text for details. G, granulosa layer; T, thecal layer; Oc, oocyte; C, cumulus granulosa cells.

confirmed by immunoblotting with antibody preabsorbed with the Cx43 peptide (Fig. 5B). Linearity of detection by densitometry was confirmed by a dose response evaluation (Fig. 5C).

Regardless of follicular size or status, granulosa cells contained threefold more ($p < 0.01$) Cx43 protein than thecal cells (10.3 ± 2.1 vs 3.5 ± 0.7 arbitrary OD units).

Across both cell types (granulosa and theca) and regardless of follicular status, large and medium follicles expressed twofold more ($p < 0.05$) Cx43 protein than small follicles (8.1 ± 1.6 vs 3.7 ± 1.1 arbitrary OD units). Across both cell types (granulosa and theca) and regardless of follicular size, healthy follicles expressed threefold more ($p < 0.05$) Cx43 than atretic follicles (9.7 ± 1.9 vs 3.2 ± 0.7 arbitrary OD units).

Overall, follicular status had an effect on expression of Cx43. Granulosa of healthy follicles contained more ($p <$

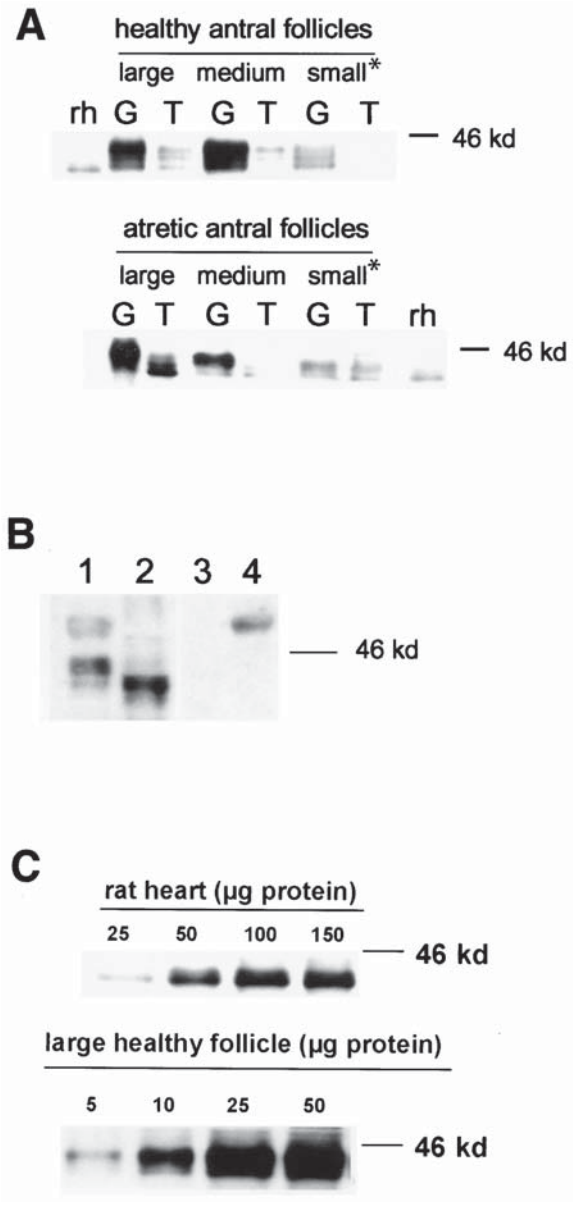

Fig. 5. (A) Western analysis of Cx43 in follicles. Representative samples of granulosa (G) and thecal (T) tissues from healthy or atretic large, medium, or small antral follicles. *, Small high progesterone follicles are included with healthy follicles and small low progesterone follicles are included with atretic follicles in this analysis. Rat heart (rh) was included in each analysis as a positive control tissue for densitometry. For large and medium healthy follicles, lanes contained 25 µg protein. For small high progesterone follicles, lanes contained 50 µg protein, and for atretic follicles and small low progesterone follicles, lanes contained 100 µg protein. All lanes were evaluated relative to rat heart and were adjusted for the amount of protein loaded (*see* Fig. 5C). **(B)** Cx43 primary antibody neutralization. Lanes 1 and 2 were immunoblotted with primary antibody to Cx43 and lanes 3 and 4 were immunoblotted with primary antibody preabsorbed with peptide as described in Materials and Methods. Lanes 1 and 4 contained 150 µg protein from a large follicle and lanes 2 and 3 contained 5 µg protein from rat heart (positive control tissue). Note the presence of specific Cx43 bands in lanes 1 and 2 and the absence of these bands in lanes 3 and 4. **(C)** Dose response for densitometry. Top lanes contained rat heart protein (positive control tissue) and bottom lanes contained protein from the granulosa tissue of a large healthy follicle. For rat heart and the large healthy follicle, detection was linear ($p < 0.01$; $r = 0.99$ and 0.98, respectively).

0.01) Cx43 protein than either granulosa of atretic or theca of healthy follicles (15.4 ± 3.2 vs 4.7 ± 1.1 or 4.9 ± 0.9 arbitrary OD units). Cx43 expression in granulosa of atretic follicles was similar ($p > 0.1$) to that of healthy or atretic thecal tissues (4.7 ± 1.1 vs 4.9 ± 0.9 or 1.7 ± 0.6 arbitrary OD units). Additionally, healthy large and medium follicles contained more ($p < 0.01$) Cx43 protein than atretic large and medium follicles or small follicles (11.3 ± 2.3 vs 3.4 ± 1.0 or 3.7 ± 1.1 arbitrary OD units). For small follicles, Cx43 expression was similar ($p > 0.1$) whether they were high or low progesterone concentration follicles (4.9 ± 2.1 vs 2.8 ± 1.1 arbitrary OD units).

Detection of Cx32 and Cx26 by Western analysis was attempted but was unsuccessful. Antibodies, obtained from several sources, were used. The specific epitope for these proteins, which were detected unambiguously by immunohistochemistry, may have been masked by binding to the nylon membrane.

Discussion

In this experiment, we have demonstrated a pattern of expression of Cx43, Cx32, and Cx26 proteins across follicular development in cows that differs in some important aspects from that of other species. The present study found Cx26 in oocytes in the earliest stage of development, the primordial follicle, a finding that has only previously been reported in sheep *(19)*. Cx32 was only found in follicles that were undergoing atresia, suggesting that its expression could be used as a "marker" for the beginning of follicle degeneration. Additionally, there were changes in the location and relative abundance of Cx43, Cx32, and Cx26 throughout follicular growth or regression. Since detection of connexins has been accepted as an indicator of the presence of structural and functional gap junctions, this is evidence that multiple connexins affect follicular development and atresia in cows *(7,8,19,23–26)*.

Recent hypotheses suggest that apoptosis or programmed cell death is either initiated or controlled by "messages" sent from cell to cell via gap junctions *(27)*. If atresia of follicles, which is a form of apoptosis, could be prevented by altering gap junctional signals for cell death, the numbers of follicles ripening to ovulation would be increased, thus increasing fertility in cows. Conversely, once we understand how gap junctions influence cell death in this model, we may be able to devise ways to shut down cell proliferation in tumors.

Cx43, Cx32, and/or Cx26 have been localized to developing follicles throughout the estrous cycle or to the cumulus-oocyte complex, but patterns of expression are different in different species *(7,28)*. In rats, Cx43 protein was

detected in the oocyte cytoplasm and/or granulosa cells of primordial and primary/secondary follicles *(29,30)*, but in cows (this study), Cx43 was only between granulosa cells and on the borders between the oocyte and granulosa cells in primary/secondary follicles and antral follicles, and not in oocytes. In this study, Cx26 protein was the only connexin present in oocytes and it was only in primordial and primary/secondary follicles, which conflicts with previous studies, which found Cx43 and Cx32 in oocytes from preovulatory follicles *(14,31)*. However, our study did not focus on the oocyte-cumulus complex from antral follicles. During the early stages of follicular development, gap junctions seem to be necessary for transfer of nutrients, but during the later stages, gap junctions also are involved in maintaining the meiotic arrest of preovulatory oocytes *(7,14–16,23,31–33)*.

By immunohistochemistry and immunoblotting, we have shown that Cx43 protein increased during growth of bovine antral follicles. Similar to reports for cyclic rats, mice, and sheep *(19,30,34,35)*, immunoblotted Cx43 protein formed multiple bands, probably reflecting a difference in phosphorylation of the Cx43 protein *(17,19,27, 30,34–38)*. Although the importance of phosphorylation in changing the characteristics of gap junctions and the rate of cell–cell communication is still not fully understood, Lenhart and coworkers *(27)* have recently reported an increase in Cx43 protein phosphorylation without a concomitant increase in Cx43 expression during porcine follicular development.

The relative abundance of Cx43 and Cx26 decreased in atretic antral follicles of cows. Cx43 was detected in theca of atretic small follicles, but not in theca of atretic medium and large follicles, a change from the pattern of Cx43 expression in healthy follicles. In atretic follicles of rats and pigs, Cx43 either was not seen or its expression was decreased in granulosa and/or thecal cells *(27,30,39)*. Similarly, in sheep, Cx43 protein was seen only occasionally in theca and not in granulosa cells of atretic follicles *(19)*. Furthermore, a decrease in expression of Cx43 during atresia was confirmed in another study, which used a hypophysectomized ewe model to induce atresia *(40)*.

The overall pattern of expression of Cx43 and Cx26 proteins in preantral and healthy antral follicles suggests that these two connexin proteins may help to maintain the health of the oocyte during follicular growth in cows through selective passage of regulatory molecules, e.g., cyclic AMP, regulators of protein kinase C, and so on, and nutrients *(2,7,19)*. However, we did not evaluate whether the Cx43 and Cx26 proteins are colocalized in gap junctional channels of bovine follicles or whether both transcripts are expressed in the same cells. In mice, both the transcripts and proteins for Cx43 and Cx32 were present in the cumulus-oocyte complex *(31)*. In future studies, it will be important to determine whether both Cx43 and Cx26 transcripts are present and if their proteins colocalize to gap junctional channels as a way of regulating follicle growth or atresia *(5,10–12)*.

In contrast to the expression patterns of Cx43 and Cx26, Cx32 was not detected in healthy follicles, but was strikingly present in granulosa of all atretic small and about half of the medium antral follicles where it was cytoplasmic rather than punctate in appearance (punctate staining indicating assembled gap junctions). In fact, the presence of cytoplasmic staining for Cx32 appeared to be a "marker" of atresia in antral follicles. These findings contrast with previous studies localizing Cx32 to the cumulus-oocyte complex of mouse and bovine preovulatory follicles *(14,31)*. It is certainly not apparent why Cx32 would be detected exclusively in atretic follicles if expression of Cx32 in later stages of follicular development is critical to ovulation.

Similar to our findings, Itahana and coworkers *(41)* have reported the differential expression of mRNA for four connexin genes, Cx43, Cx32, Cx30.3, and Cx26, in the porcine ovary and follicles. Additionally, they have isolated a new connexin, Cx60, in porcine follicles, that was expressed in thecal and cumulus cells and was thought to have a role in preovulatory or ovulatory processes *(42)*. In Cx37-deficient mice, there are no mature (Graafian) follicles, ovulation does not occur, and formation of corpora lutea is inappropriate *(43)*. These experiments and the present study reinforce the notion that expression of multiple connexins may be a mechanism of regulating development and regression of follicles.

Materials and Methods

Tissue Collection

For both experiments, whole ovaries from the mid- or late-luteal phase of the estrous cycle (determined as previously described; *44*) were obtained from a local slaughterhouse and transported back to the laboratory on ice. For experiment 1, ovaries ($n = 24$ from 20 cows) were flushed via the main ovarian artery with PBS (0.01 *M* phosphate, 0.14 *M* NaCl, pH 7.3), containing 0.1% lidocaine, to remove blood cells and dilate the ovarian vascular bed, and then were perfused with Carnoy's solution to preserve the integrity of the follicles *(45)*. After perfusion, follicles were cut longitudinally through their mid section, so that small, medium, and large antral follicles could be identified, and the ovarian pieces were fixed further by immersion in Carnoy's for 4 h, as previously described *(45)*. Fixed ovarian pieces were dehydrated, and paraffin-embedded sections were cut at 8 μm to help maintain the integrity of the larger follicles and provide a more accurate estimate of numbers of primordial follicles *(46)*. Sections were mounted onto positively charged glass slides (FisherBiotech ProbeOn Plus; Fisher Scientific, Pittsburgh, PA) and used for immunohistochemical localization of connexins. For experiment 2, ovaries ($n = 29$ ovaries from 25 cows) were examined and the surface diameter of follicles was measured to identify

small (<5 mm), medium (5–10 mm), and large (≥10 mm) antral follicles. Follicular fluid from each follicle was aspirated and frozen until evaluation of estradiol and progesterone concentrations by radioimmunoassay (RIA) was performed. Granulosa and thecal layers were dissected as described previously *(47)* and snap-frozen separately in liquid nitrogen for Western analysis. For large and medium follicles each sample represented one follicle, but for small follicles each sample represented two to three follicles pooled after determining follicular fluid estrogen and progesterone concentrations.

Immunohistochemistry (Experiment 1)

After fixation and treatment with blocking buffer (PBS + Triton-X100 [0.3% vol/vol] + normal goat serum [1–2% vol/vol]), sections were incubated overnight at 4°C with a 1:25 dilution of rabbit polyclonal antibody against Cx43, Cx32, or Cx26 (Zymed Laboratories Inc., San Francisco, CA). A biotinylated secondary antibody (goat antirabbit IgG; Vector Laboratories, Burlingame, CA), the ABC Vectastain Elite kit, and the substrate Vector SG were used to detect the Cx43, Cx32, or Cx26 primary antibody. For control staining, normal rabbit serum (used at the same dilution) replaced the primary antibody. Additionally, the primary antibody which was preabsorbed with its specific peptide (Zymed) and incubated (1:25 dilution) with tissue sections, also demonstrated the absence of specific staining that was shown by control staining (data not shown).

For immunohistochemical evaluations, antral follicles were measured and classified after adjusting the measurements with a shrinkage factor to account for dehydration and embedding, as we have previously reported *(48)*. The overall pattern of staining was evaluated and the percentage of follicles that contained a particular connexin protein within a specific compartment of the follicle was recorded. Follicles were classified as healthy or atretic on the basis of their morphology *(45,49)*. Overall, 27 sections were evaluated, and these contained >130 primordial follicles, 101 primary/secondary follicles, and >100 antral follicles.

Protein Extraction (Experiment 2)

To optimize protein extraction from granulosa and thecal tissues, total proteins were isolated from follicular tissues using a method for extraction of proteins from cultured cells proposed by Wang and coworkers *(50)* with modifications. This method eliminates most DNA, prevents endogenous protease activity, and is compatible with protein assay, gel electrophoresis, and Western analysis *(50)*. Homogenization buffer was 2% SDS, 5 m*M* EDTA, 5 m*M* EGTA, 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL 4-(2-aminoethyl)-benzenesulfonylfluoride [AEBSF], 5 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL *N*-α-*p*-tosyl- L-lysine chloromethyl ketone [TLCK], 10 μg/mL *N*-α-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 20 m*M* HCl (pH 7.4). Tissues were homogenized briefly using a polytron (Brinkman Instruments, Westbury, NY) followed by sonication with an ultrasonic processor (Sonics & Materials, Danbury, CT). Proteins were then precipitated by adding one part 100% trichloroacetic acid (TCA) to three parts homogenate. After centrifugation (3600*g* for 5 min), pellets were washed with 2.5% TCA and recentrifuged. Pellets were resuspended in distilled water and adjusted to pH 7.5 with 3 *M* Tris base to redissolve proteins. Protein solutions that did not appear to be completely solubilized after pH adjustment were clarified by another brief sonication. Protein concentrations of these resuspended protein solutions were determined by using the Coomassie Brilliant Blue G assay with BSA (fraction V; Sigma Chemical Co., St. Louis, MO) as the standard *(25,51,52)*.

Western Immunoblot Analysis (Experiment 2)

Protein solutions of homogenized follicular tissues were subjected to SDS-PAGE as we have previously reported *(25)*. A protein solution of homogenized rat heart was used as a control and standard in each analysis. Samples were added to loading buffer [glycerol (10%, vol/vol), SDS (2% vol/vol), 2 m*M* EDTA, β-mercaptoethanol (1%, vol/vol), bromophenol blue (0.05%, wt/vol), Tris-HCl (63 m*M*), pH 6.8], boiled for 2 min, and then applied to a 12% polyacrylamide gel with a 3% stacking gel *(53)*. Electrophoresis was conducted in buffer [Tris (50 m*M*), glycine (383 m*M*), SDS (0.1%), EDTA (0.4 m*M*)] for 18 h at constant current *(23)*. Separated proteins were electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA) in buffer (methanol [20%, vol/vol], Tris [20 mM], glycine [150 m*M*]) for 3 h at constant current. After blocking the membrane in buffer (PBS with Tween-20 [0.1%, vol/vol]) containing dried milk (10% wt/vol; OurFamily, SunMart) for ~4 h at room temperature with agitation, the membrane was incubated with a polyclonal antibody against Cx43 (Zymed) at 1:500 in the same buffer with 5% milk at 4°C overnight. The membrane was then incubated with a peroxidase-labeled antirabbit antibody (Amersham Corporation, Arlington Heights, IL; 1:4000) in buffer containing 5% milk for 1 h, followed by detection by using ECL reagents (Amersham) and autoradiography. To check the specificity of antibody binding, Cx43 antibody was preabsorbed with tenfold excess of specific Cx43 peptide (Zymed) and used for analysis as described above. To check the linearity of detection of Cx43 proteins, dose response measurements were conducted for rat heart (control) proteins and proteins from one large follicle. For each measurement of optical density (OD) units, sample values were normalized to the measurements of rat heart included in each analysis. When necessary, a second OD measurement of a sample was made using less protein/lane to avoid overexposure during autoradiography.

RIA for Estradiol and Progesterone (Experiment 2)

Estradiol and progesterone concentrations in unextracted follicular fluid were measured by RIA using methods vali-

dated in our laboratory *(47)*. Assay sensitivity for estradiol was 1 pg/tube and for progesterone was 25 pg/tube. All samples were assayed in a single assay; the intra-assay coefficient of variation was 13.3% for estradiol and 11.2% for progesterone. Large and medium follicles were classified as healthy when the follicular fluid estradiol: progesterone (E:P) ratio was ≥1, and as atretic when E:P ratio was <1 *(47,54)*. Since the estradiol concentration in the follicular fluid of small follicles was below the limit of assay sensitivity, we classified small follicles on the basis of high (>60 ng/mL) or low (<10 ng/mL) follicular fluid progesterone concentration.

Statistical Analysis (Experiment 2)

Data were analyzed as a split-plot design by using the general linear models ANOVA, with follicle size, follicle status (healthy or atretic), and cell type (granulosa or theca) and all their interactions included in the model *(55)*. When the F test was significant, differences between specific means were evaluated by using Bonferroni's multiple comparison procedure *(56)*. Data are expressed as means ± SEM unless noted otherwise.

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