Endocrine, *vol. 8, no. 3, 269–279, June 1998* 0969–711X/98/8:269–279/$10.75

Gap Junctional Proteins, Connexin 26, 32, and 43 in Sheep Ovaries Throughout the Estrous Cycle

Anna T. Grazul-Bilska,[1,2] Dale A. Redmer,[2] Jerzy J. Bilski,[1,2]
Albina Jablonka-Shariff,[2] Vinayak Doraiswamy,[2] and Lawrence P. Reynolds[1,2]

[1]Cell Biology Center, Biotechnology Institute
and [2]Department of Animal and Range Sciences, North Dakota State University, Fargo, ND

Ovarian follicles from days 13, 14, 15, and 16 and corpora lutea (CL) from days 2, 4, 8, 12, and 15 of the estrous cycle were evaluated for the presence of connexins by immunohistochemistry. In addition, CL from days 5, 10, and 15 of the estrous cycle were used for immunofluorescent detection of Cx43 followed by image analysis, and for Western immunoblot. In all tissues, staining for all connexins appeared punctate, indicating the presence of assembled gap junctions. Cx26 was present in the ovarian surface epithelium, stroma, and blood vessels within the stroma and hilus, and in the CL. In healthy antral follicles, Cx26 was present only in the theca layer, whereas Cx43 was present in granulosa and theca layers. In the majority of atretic follicles, connexins were not detected, but in 13% of the atretic follicles, Cx43 was present in the theca layer. Cx32 was detected in the blood vessels of ovarian stroma and in the CL, and Cx43 was detected in the CL. Localization and/or expression of connexins depended on stage of luteal development. Western analysis demonstrated that expression of Cx32 in luteal tissues was similar across the estrous cycle. The area of positive staining for Cx43 and expression of Cx43 in luteal tissues decreased ($p < 0.05$) as the estrous cycle progressed. The pattern of expression of connexins indicates that gap junctional proteins may be important in the regulation of folliculogenesis and follicular atresia, as well as growth, differentiation, and regression of the CL.

Key Words: Gap junctions; Cx26; Cx32; Cx43; follicles; corpora lutea; ovaries; ewe.

Received January 6, 1998; Revised February 18, 1998; Accepted March 5, 1998.

Author to whom all correspondence and reprint requests should be addressed: Dr. Anna T. Grazul-Bilska, Cell Biology Center, 119b Hultz Hall, North Dakota State University, Fargo, ND 58105. E-mail: grazul@plains.nodak.edu
Current Address: Dr. Albina Jablonka-Shariff, Dept. Obstetrics/Gynecology, Washington University School of Medicine, St. Louis, MO 63110.

Introduction

Ovarian follicles and corpora lutea (CL) are the most rapidly growing normal tissues in adults that grow, differentiate, and regress during each estrous cycle and/or pregnancy *(1–4)*. Successful development of follicles to the preovulatory stage and subsequent development of the CL require cellular interactions among ovarian cells to coordinate the functions of these diverse cell types that are proliferating and differentiating extremely rapidly *(1–7)*. Such cellular interactions may be mediated by several mechanisms, including humoral pathways as well as the contact-dependent gap junctional pathway *(8)*.

Gap junction-mediated intercellular communication has been shown to be important for coordination of cellular metabolism and function during growth and differentiation of organs and tissues *(9–13)*. Gap junctions are important in signal transduction, and in transport of nutrients within avascularized or poorly vascular tissues, such as the follicular granulosa layer or corpora hemorrhagica, since gap junctional channels allow for transfer of molecules <1000 M_r *(7,8,12,14–20)*. Thus, during follicular development, gap junctions are thought to be involved in regulating differentiation and maturation of the cumulus–oocyte complex *(21–23)*. During development of CL, gap junctions mediate cellular interactions, which may be important for progesterone production and the luteolytic process *(8,15)*.

Gap junctions are composed of hemichannels (connexons), which consist of six-subunit proteins named connexins *(9,24)*. Distribution of connexins is tissue- and cell-specific *(9,24)*. Several connexins have been identified within ovarian tissues of numerous species *(5,8,14,15,18,21,25–37)*.

The aim of the present study was to evaluate the presence of Cx26, Cx32, and Cx43 in ovine ovaries at several stages of follicular and luteal development.

Results

Immunohistochemical staining demonstrated punctate staining for Cx26, Cx32, and Cx43 in several compartments of ovine ovaries throughout various stages of the

Table 1
Localization of the Gap Junctional Proteins Connexin (Cx) 26, Cx32, and Cx43 in Ovine Ovaries[a]

Ovarian compartment	Protein
Surface epithelium	Cx26
Follicles	
Primordial and primary	Cx26
Secondary	Cx43
Antral	
Healthy	Cx26 in T[b]
	Cx43 in T and G[c]
Atretic	Cx43 in T
Corpora lutea	
Connective tissue	Cx26 and Cx32
Blood vessels	Cx26, Cx32, and Cx43
Parenchyma	Cx26, Cx32, and Cx43
Ovarian blood vessels	Cx26 and Cx32
Connective tissue	Cx26 and Cx32

[a]This table has been published as preliminary observation in Grazul-Bilska et al. *(15)*.
[b]T, theca cells
[c]G, granulosa cells

estrous cycle, and the location and type of connexin are summarized in Table 1. Cx26 was present in the theca layer of healthy small, medium, and large antral follicles, in the surface epithelium of the ovary, in the stroma of the cortex, and in blood vessels throughout the ovary, including the hilus (Fig. 1A,B). Weak staining of Cx26, but not Cx32 or Cx43 appeared in primary follicles (data not shown). In the CL, Cx26 was detected in the connective tissue and blood vessels within the luteal capsule, in the connective tissue tracts between the parenchymal lobules, in the interstitial areas of the parenchymal lobules, and in the parenchymal cells throughout the estrous cycle (Fig. 1C,D). At the early luteal phase (days 2 and 4), Cx26 was present in the connective tissue capsule and connective tissue tracts between parenchymal lobules (Fig. 1C). At the mid- and late-luteal phases of the estrous cycle (days 8, 12, and 15), Cx26 was localized homogeneously throughout the luteal parenchyma and, in addition, was present in the cytoplasm of some luteal cells (Fig. 1D).

Cx32 was detected in the blood vessels of the ovarian stroma in the cortex and in luteal tissues throughout the estrous cycle (Fig. 2A–D). Week staining for Cx32 was also observed in the ovarian connective tissues (data not shown). Luteal Cx32 was present exclusively in the parenchymal lobules on day 2 (Fig. 2B). By day 4 of the estrous cycle, staining also was observed in the connective tissue tracts between the parenchymal lobules (Fig. 2C). Punctate staining was located at the borders between adjacent luteal cells and also in the cytoplasm of some of the luteal cells (Fig. 2B,C). By midcycle, Cx32 was primarily present in some luteal blood vessels and connective tissues (Fig. 2D), and rarely within parenchymal cells (not shown).

Cx43 was present in all sizes of follicles, except primordial and primary (Fig. 3A). In secondary follicles, Cx43 was localized to the granulosa, but not the theca layer and in the area of contact between the granulosa cells and the oocyte (Fig. 3B). In healthy small, medium, and large antral follicles, Cx43 was present in granulosa and theca layers (Fig. 3C,D). However, in healthy antral follicles, staining was more dense in the granulosa compared with the theca layer (Fig. 3C,D). In addition, positive Cx43 staining was more intense in large follicles compared with small or medium follicles (Fig. 3C,D). Positive staining for Cx43 was not detected in the majority (83%) of small, medium, or large atretic follicles (n = 72). However, Cx43 was detected in the theca layer of 13% of the small and medium atretic follicles (Fig. 3E). These follicles appeared to be at a relatively early stage of atresia *(3,38)*. In all classes of follicles, staining was punctate and/or linear and localized on the cellular borders.

Cx43 was localized on the cellular borders between luteal parenchymal cells throughout the estrous cycle (Figs. 4 and 5). The distribution of Cx43 positive staining in luteal tissues was relatively homogenous on days 5 and 10 (Figs. 4 and 5A,B), but heterogenous (i.e., some areas were stained, whereas others were not stained) on day 15 of the estrous cycle (Fig. 5C).

In agreement with the descriptive evaluation, image analysis indicated that in luteal tissues, the area of positive staining for Cx43 was greatest ($p < 0.05$) on day 5 ($0.15 \pm 0.01\%$ of total area), then decreased ($p < 0.05$) on day 10 ($0.08 \pm 0.001\%$), and was the least ($p < 0.05$) on day 15 ($0.007 \pm 0.001\%$) of the estrous cycle.

Preadsorbtion of the antibodies with their corresponding connexin peptide resulted in a complete lack of staining for Cx26, Cx32, or Cx43. Figure 6 demonstrates an example of staining for Cx26 with or without preadsorbed antibody.

Western immunoblot analysis for Cx32 revealed a single band at 32 kDa, and for Cx43 revealed bands in the range of 43–46 kDa for luteal tissues from days 5, 10, and 15 of the estrous cycle (Fig. 7A,B). For mouse liver lysate used as a positive control in the Cx32 assay, two bands at 27 and 45–50 kDa were revealed (data not shown) as demonstrated for mouse and rat liver, and other tissues by others *(39–41)*. For rat heart used as a positive control in the Cx43 assay, bands in the range of 43–46 kDa were present (data not shown). Expression of Cx32 was similar across the estrous cycle, but expression of Cx43 was greatest ($p < 0.05$) on day 5, decreased ($p < 0.05$) on day 10, and was the least ($p < 0.05$) on day 15 of the estrous cycle (Fig. 7C). Cx26 was not detected in luteal tissues by Western immunoblot with polyclonal antibodies or monoclonal antibodies (MAbs), but was detected in mouse liver lysate, which was used as a positive control (data not shown).

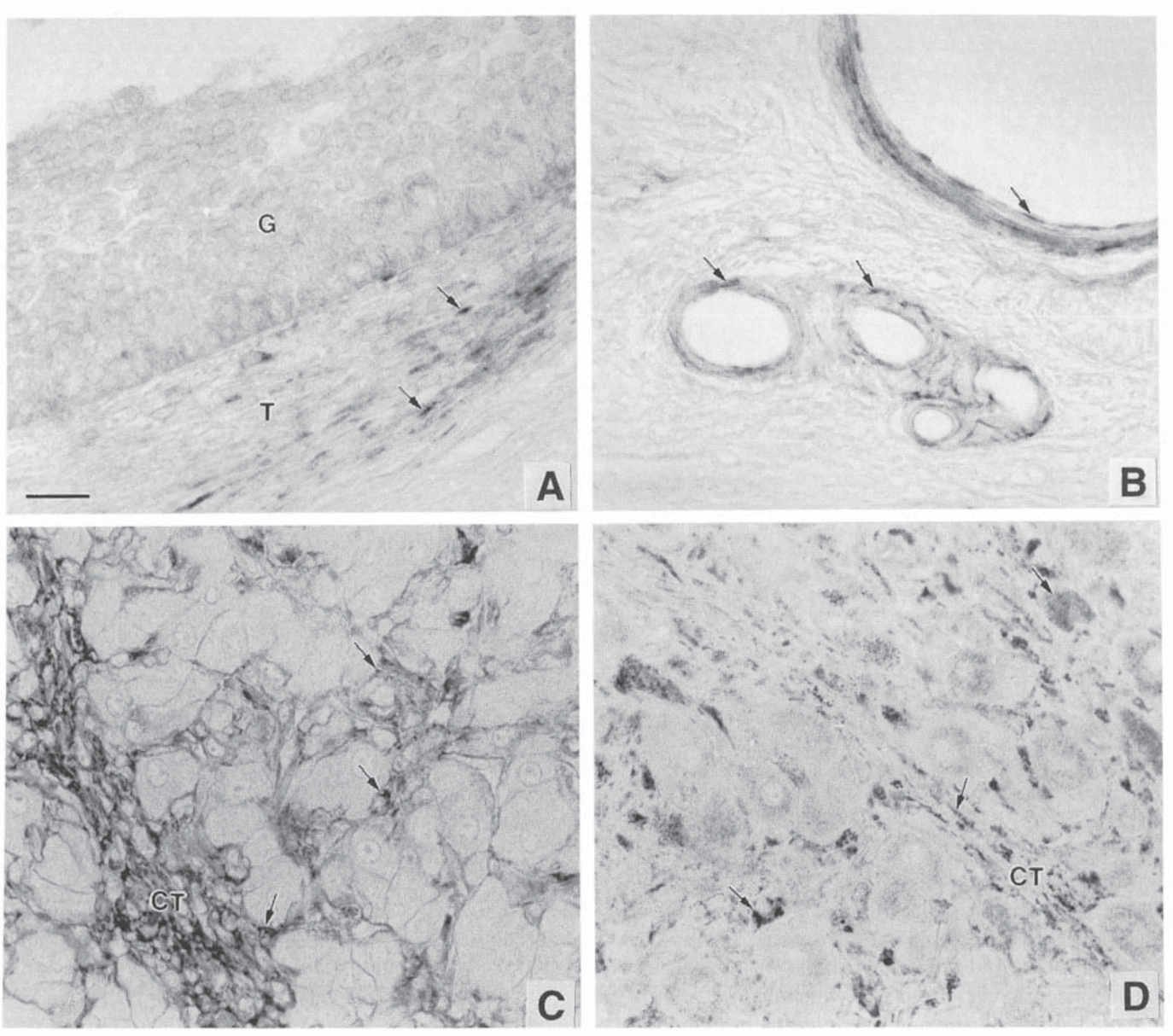

Fig. 1. Localization of Cx26 in **(A)** a healthy small antral follicle, **(B)** several sizes of blood vessels, **(C)** a day 4 CL, and **(D)** a day 15 CL. Control sections did not exhibit any positive staining (*see* Figs. 4C and 6B). Note punctate staining (A) in the theca (T) but not the granulosa (G) layer, (C) within connective tissue (CT) tracts, and (D) within connective tissue tracts and in some luteal cells (arrows). Bar = 20 μm.

Discussion

Data from the present experiment demonstrate localization of Cx26, Cx32, or Cx43 in ovarian follicles, CL, blood vessels, and stromal tissues throughout the estrous cycle. Detection of gap junctional proteins has been widely used as an indicator of the presence of structural and functional gap junctions *(5,8,14,16,18,19,25,30,42,43)*.

Former studies demonstrated the presence of gap junctions in ovarian follicles and luteal tissues of several species by electron microscopy (for review, *see 8,15*). The presence of functional gap junctions also has been reported for granulosa cells, theca cells, and luteal cells of several species *(5,8,14–16,33,44–49)*. Recent studies also have demonstrated the expression of several connexins (Cx26, Cx30.3, Cx32, Cx40, Cx43, Cx45, Cx60) in ovarian follicles and/or CL of various species *(5,8,14,15,18,19,28,30–37)*.

The pattern of expression of Cx26 and Cx43 in healthy primary, secondary, and antral follicles of sheep was similar to the pattern previously reported for gap junctions or connexin expression observed for rat, rabbit, bovine, or porcine follicles *(21,29–36,50,51)*. In contrast with the present data, however, gap junctions and Cx43 were detected in primordial follicles of rats *(29,30,51)*. Staining for Cx43 was more dense in the granulosa compared with

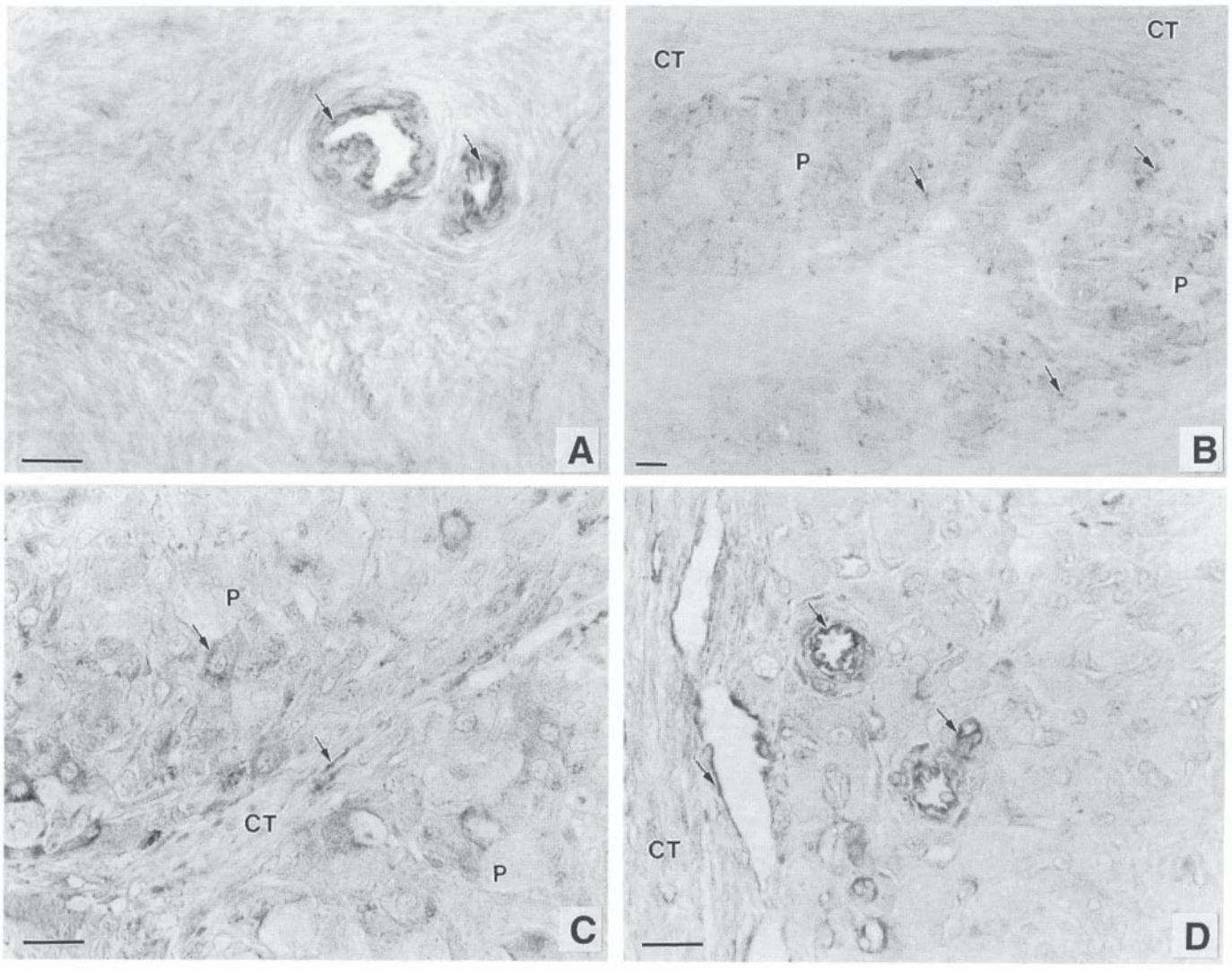

Fig. 2. Localization of Cx32 in **(A)** blood vessels, **(B)** day 2 CL, **(C)** day 4 CL, and **(D)** day 8 CL. Control sections did not exhibit any positive staining (*see* Figs. 4C and 6B). Note punctate staining (B) only within the luteal parenchymal lobules (P), (C) in the parenchyma (P), and in the connective tissue (CT) tracts between the parenchymal lobules, and (D) in blood vessels and connective tissue of the luteal capsule (arrows). Bar = 20 μm.

the theca layer for all classes of healthy follicles, and also appeared to be more intense in large than medium or small follicles. Therefore, this pattern of staining is comparable with the pattern of Cx43 expression in ovarian follicles in cows *(34)*, and demonstrates the effect of stage of follicular development on Cx43 expression.

In the present study, Cx32 was not detected in ovine follicles from several stages of follicular development. This indicates that Cx32 may not be involved in folliculogenesis in sheep. However, Cx32 was detected in the oocyte of bovine preovulatory follicles *(28)* and in cumulus–oocyte complex in mice *(23)*, but our study did not focus on cumulus–oocyte complex.

In atretic follicles, connexins were not detected in granulosa cells, and Cx43 was rarely seen in the theca layer. Similarly, in rats and pigs, Cx43 was not expressed in granulosa or theca layers of atretic follicles *(29,36)*. In contrast, other studies have reported that Cx43 was present in theca and/or granulosa layers of rat and bovine atretic follicles *(30,35)*. These data suggest that gap junctions may play a role in the process of atresia.

The current data also demonstrated that Cx26, Cx32, and Cx43 were present in ovine luteal tissues, and the pattern of staining changed throughout the estrous cycle. Cx26, Cx32, and Cx43 also are present in bovine CL across the estrous cycle; however, the pattern of staining for Cx26 and Cx32 differs from ovine CL *(8)*.

The pattern of Cx43 expression in sheep CL, greatest in the early and midluteal phases and least in the late-luteal phase, was similar to previous reports for bovine and baboon CL *(14,19)*. For porcine CL, it has been demonstrated that expression of Cx43 mRNA was high during early luteal development and in corpora albicanta, and

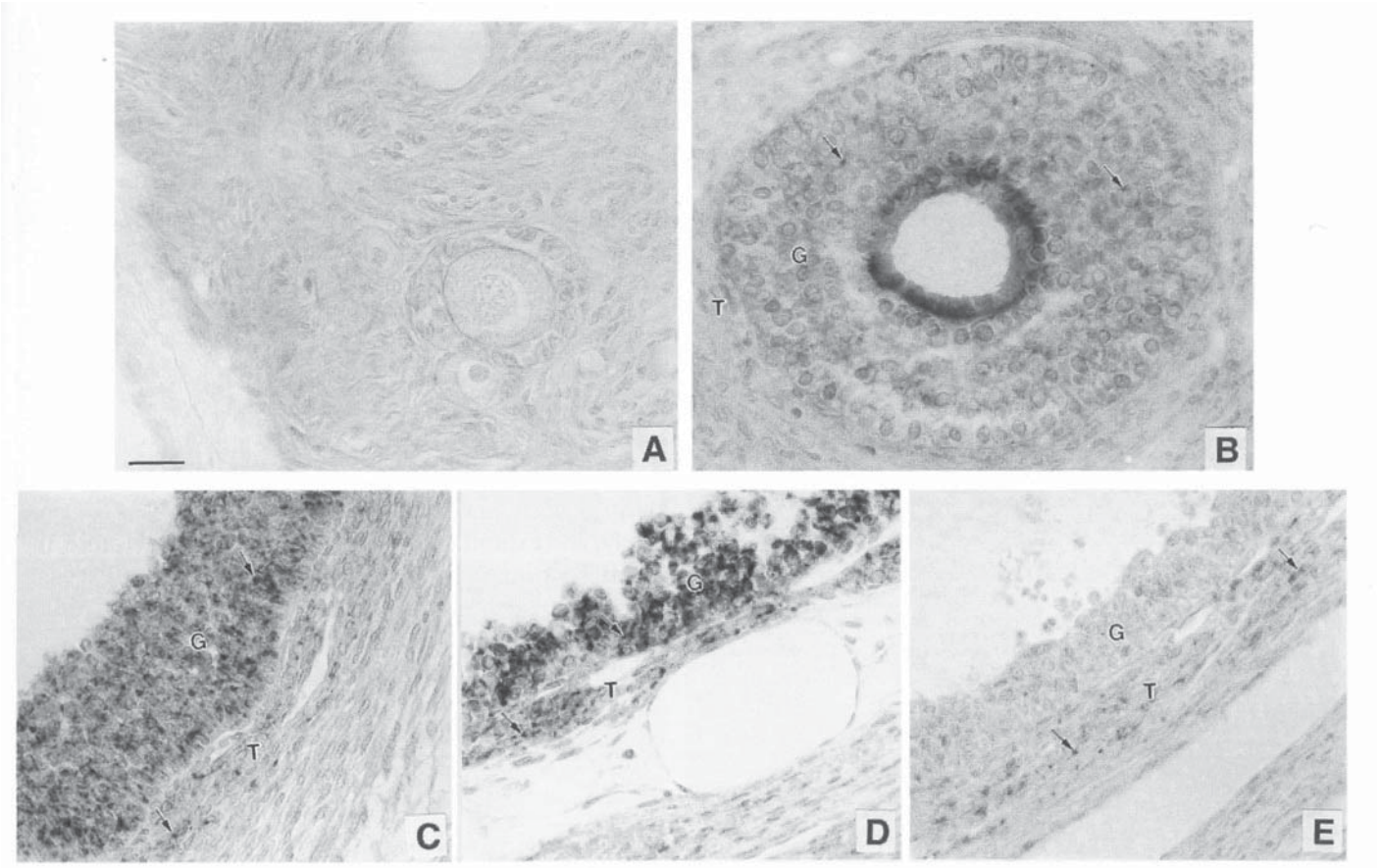

Fig. 3. Localization of Cx43 in **(A)** primordial and primary follicles, **(B)** secondary follicle, **(C)** healthy small antral, **(D)** healthy large antral, and **(E)** atretic antral follicles. Control sections did not exhibit any positive staining (*see* Figs. 4C and 6B). Note lack of staining in (A). Also note punctate staining in (B) only within the granulosa (G) layer and between the granulosa and the oocyte, in (C) and (D) within granulosa and theca (T) layers, and in (E) only within the theca layer (arrows). Bar = 20 μm.

lower in mature and regressing CL *(31)*. The highest expression of Cx43 observed during the early luteal phase parallels the greatest rate of luteal cell proliferation *(2,52)*, and suggests a relationship between gap junctions and the rate of luteal growth. In support of this suggestion, highly proliferating embryonic and postnatal tissues posses abundant structural and functional gap junctions *(17,53–55)*. Although suppression of gap junctions in growing embryonic tissues causes several abnormalities *(55)*, similar data are not available for developing follicles or CL.

Nevertheless, taken together, these data support the hypothesis that gap junctions play a role during tissue growth in ovarian follicles and CL *(10–13)*. Cellular interactions within ovarian tissues in the process of signal transduction, transfer of nutrients, production and/or secretion of steroids, or angiogenesis may be mediated by contact-dependent or contact-independent mechanisms *(5,7,8,14–16,20,43,56–58)*. It seems very likely that cellular interactions mediated by gap junctions are critical for normal ovarian function. The majority of tissues express multiple connexins *(10–13)*. Yamasaki and Naus *(13)* proposed that the presence of multiple connexin species may be important for growth control. Therefore, it also is very likely that in the ovarian tissues, which exhibit periodical growth and regression during each estrous cycle, multiple connexins are involved in the control of growth of follicles and CL.

In addition to playing a role during luteal development, gap junctions also may coordinate cellular functions in mature and regressing CL *(5,8,15)*. Corpora lutea consist of several types of cells, including steroidogenic small and large luteal cells and nonsteroidogenic cells (endothelial cells, fibroblasts, pericytes and others; *59,60*). It has been demonstrated that these cells interact through contact-dependent and contact-independent mechanisms to regulate progesterone production and luteolysis *(5,7,8,15,56,57, 59–62)*. In the present experiment, connexins were detected on the cellular borders in developing, mature and regressing CL, which suggests that the intercellular signals may be transferred among luteal cells through gap junctional channels, a concept that is supported by our previous studies of cultured luteal cells and tissues in sheep and cows *(5,8,14–16,44–46)*.

Cx26 and Cx32 were detected in the ovarian blood vessels (Figs. 1C and 2A), and Cx43 appeared to be localized between capillary endothelial cells within luteal tissues in the present study and also previous studies (Fig. 4B; *8,15*),

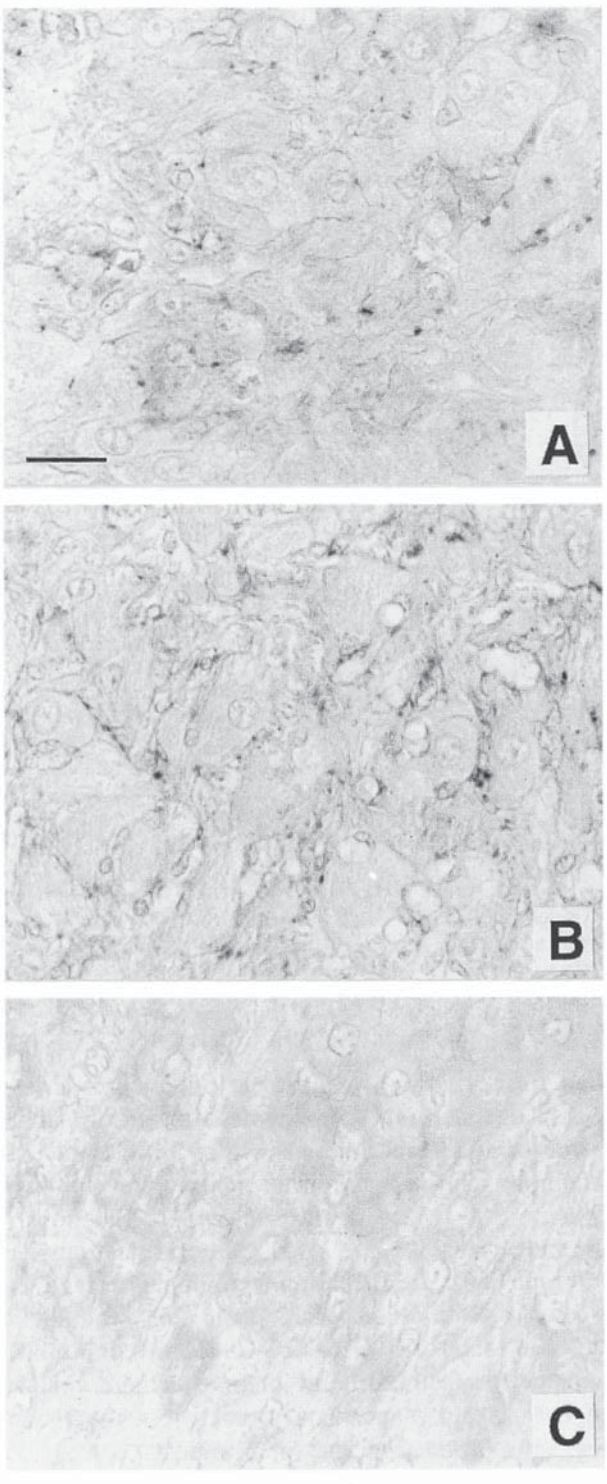

Fig. 4. Localization of Cx43 in luteal tissues from **(A)** day 5 and **(B)** day 10 of the estrous cycle. Note punctate staining on the cellular borders. Control sections, in which the primary antibody was replaced by rabbit serum, did not exhibit any positive staining **(C)**. Bar = 20 μm.

which suggests that gap junctions also may play a role in ovarian vascular cell function. Gap junctions have been demonstrated among endothelial cells in several tissues *(63,64)*. In endothelial cells, gap junctions may provide a mechanism for coordinating endothelial migration and proliferation during angiogenesis *(64)*. It has been demonstrated that luteal endothelial cells proliferate rapidly throughout the estrous cycle and pregnancy in several species *(2,4,7)*. In addition, several studies have demonstrated interactions between parenchymal and endothelial cells in CL of cows and sheep *(4,7,15,56,62)*. Therefore, gap junctions among endothelial cells may be critical for coordination of growth of blood vessels during follicular or luteal development.

Although the precise role of gap junctions in the ovaries has yet to be defined, it seems that Cx32 is not critical for ovarian functions, since Cx32-deficient mice are fertile and normal *(65)*. However, Cx26- and Cx43-deficient mice die during early embryonic or perinatal development, respectively, and exhibit numerous abnormalities. Therefore, the role of Cx26 and Cx43 in ovarian function cannot be evaluated in these gene knockout mice models *(10,66)*. Another connexin, Cx37, has recently been shown to be critical for normal follicular and luteal development in mice *(67)*. Future studies should be undertaken to evaluate how specific gap junction proteins may affect development and/or function of ovarian follicles or corpora lutea. Recently, it has been demonstrated that bovine adrenal cells, when transfected with Cx43 antisense DNA, decreased steroidogenic response to ACTH treatment *(68)*. Similarly, our studies have shown that bovine luteal cells transfected with Cx43 antisense DNA produced less progesterone in the presence of LH than nontransfected cells *(69)*. These data suggest that Cx43 is involved in the control of steroidogenesis. A closer examination of the role of gap junctional proteins in ovarian function will lead to better understanding of the mechanisms that control reproduction in sheep.

Materials and Methods

Protocols for this study and animal care were approved by the Institutional Animal Care and Use Committee.

Tissue Collection

In experiment 1, ovaries were obtained from crossbred ewes that had exhibited at least one estrous cycle of normal duration (15–18 d). Day 0 of the estrous cycle (standing estrus) was determined by using vasectomized rams. On days 13, 14, 15, and 16 (n = 4–7 ewes/d) of the estrous cycle, ovaries were removed after slaughter, the number and surface diameter of all visible follicles were recorded, and antral follicles were classified as follows: small ≤3 mm; medium, >3 mm to ≤6 mm; large, >6 mm *(3)*. To flush blood cells and dilate the ovarian vascular bed, ovaries were perfused with PBS containing 0.1% (v/v) lidocaine via the main ovarian artery *(3)*. Ovaries then were fixed by vascular perfusion of Carnoy's solution. Each fixed ovary was

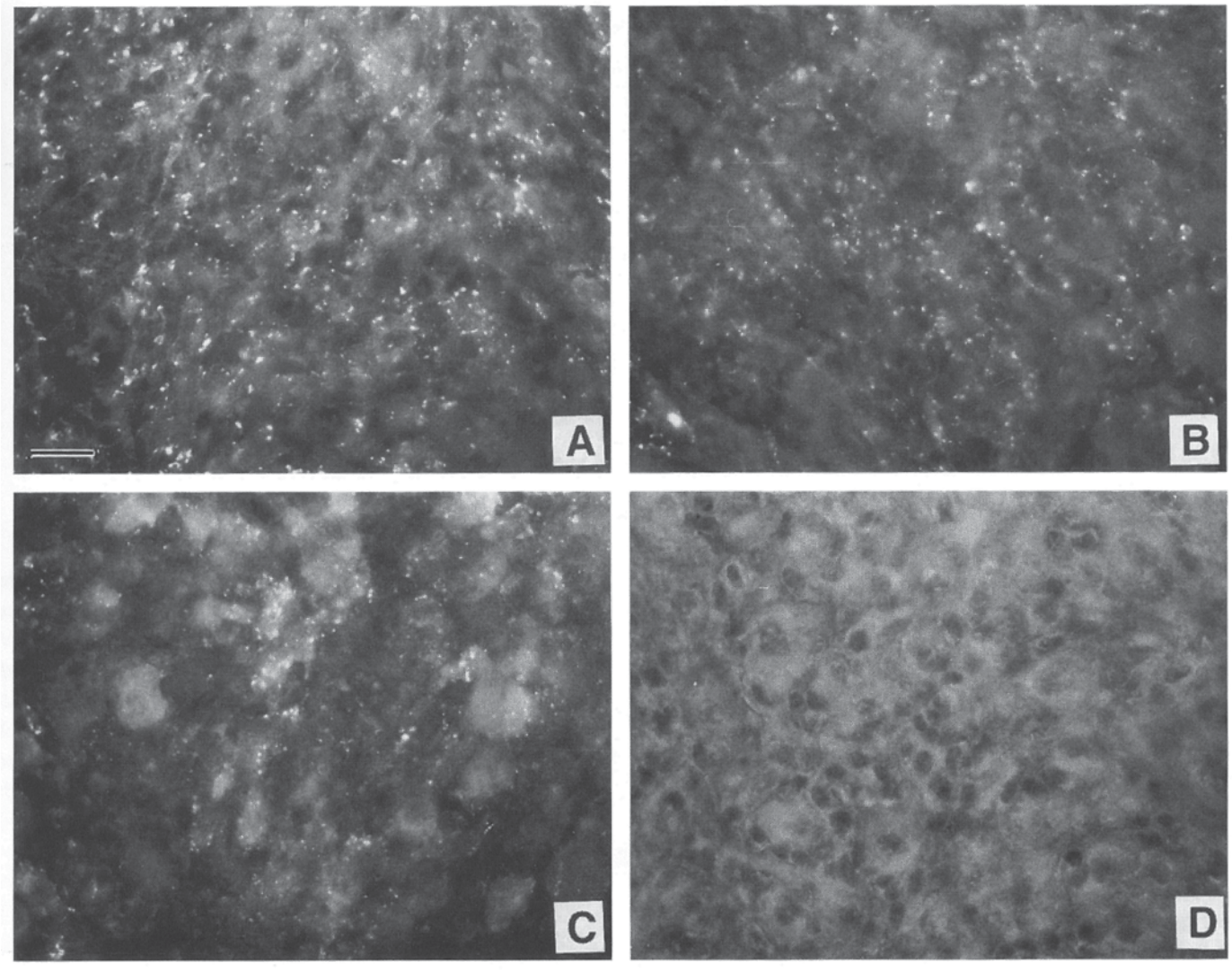

Fig. 5. Immunofluorescent localization of Cx43 in luteal tissues from **(A)** day 5, **(B)** day 10, and **(C)** day 15 of the estrous cycle. **(D)** Represents control staining, in which the primary antibody was replaced by rabbit serum. Note that staining for Cx43 exhibited brightly fluorescent punctate areas. Also note that the distribution of staining was relatively homogeneous on day 5 (A) and day 10 (B); in contrast, the distribution of staining was heterogeneous on day 15 (C), with some areas exhibiting little or not staining. Bar = 20 μm.

cut longitudinally into two or three pieces (≈5 mm thick), and immersed in Carnoy's solution for 4 h and then dehydrated, embedded in paraffin, sectioned (6 μm), and mounted onto glass slides *(2,3)*. The number of follicles analyzed for the presence of connexins is presented in Table 2.

In experiments 2 and 3, ovaries were obtained from ewes as in experiment 1. In experiment 2, CL from days 2, 4, 8, 12, and 15 (n = 4–5 ewes/d) of the estrous cycle were enucleated from the ovaries, and a sample (≈100 mg) of each CL was fixed in Bouin's solution and prepared for immunohistochemical staining *(2,3)*. In experiment 3, CL from days 5, 10, and 15 (n = 5 ewes/d) of the estrous cycle were obtained, frozen in liquid propane or in liquid nitrogen, and then used for immunofluorescent detection of Cx43 followed by image analysis or for Western immunoblot, respectively.

Immunohistochemistry

The presence of connexins in tissue sections was visualized by using immunohistochemical or immunofluorescence methods *(2,3,14)*.

For immunohistochemical detection of connexins, sections of ovarian tissues were treated for 20 min with blocking buffer consisting of PBS containing 0.3% (v/v) Triton X-100 (Malinckrodt, Paris, KY) and 1% (v/v) normal goat serum (Vector Labs. Burlingame, CA) followed by overnight incubation at 4°C with rabbit polyclonal antibodies against Cx26, Cx32 (Zymed, San Francisco, CA) or Cx43 (Zymed, which was used for staining of the sections of whole ovaries; or an antibody that was a gift from E. M. Hendrix and W. J. Larsen, University of Cincinnati, OH [as described by Hendrix et al. *{40}*], which was used for luteal tissues staining). Detection of the primary antibodies was accom-

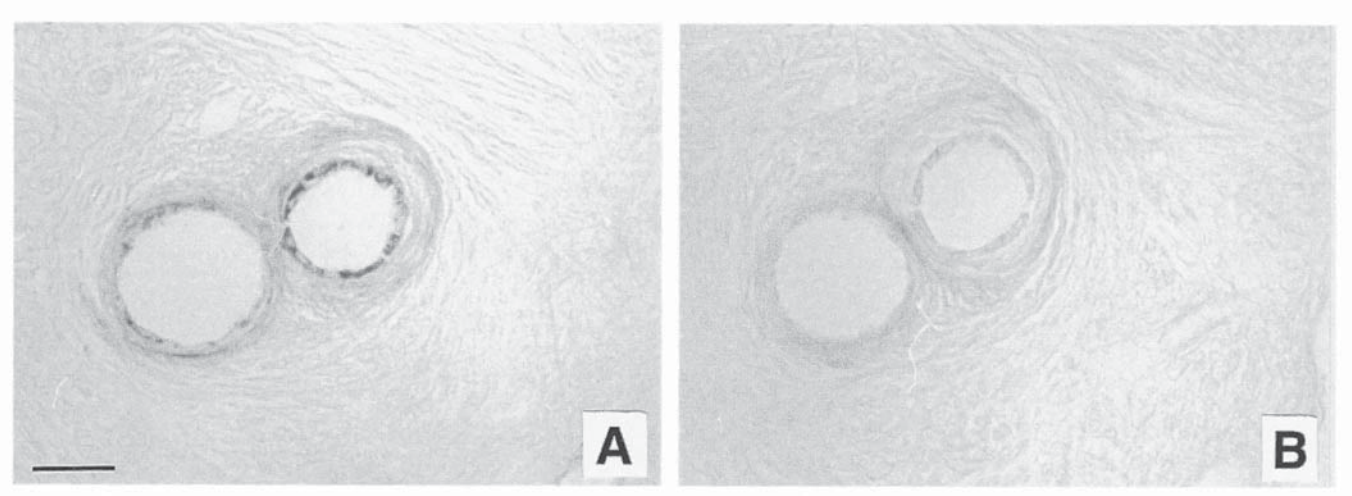

Fig. 6. Localization of Cx26 in the ovarian blood vessels by using **(A)** nonimmunoneutralized or **(B)** immunoneutralized antibody. Bar = 20 μm.

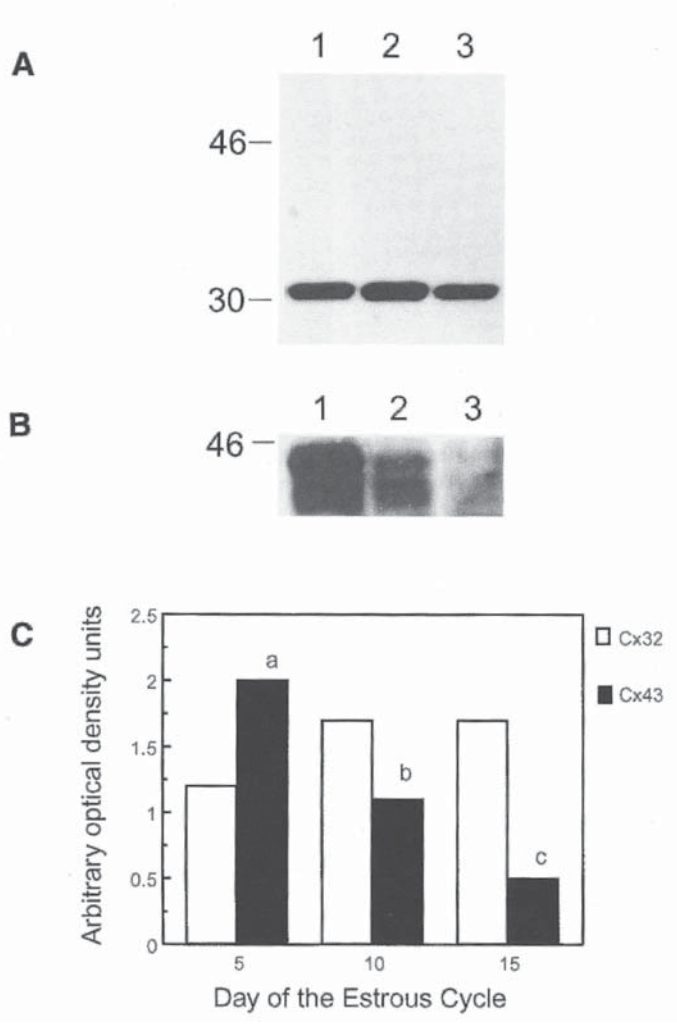

Fig. 7. Western analysis of Cx32 **(A)** and Cx43 **(B)** in ovine CL from days 5, 10, and 15 of the estrous cycle, and densitometric evaluation of Western analysis of luteal Cx32 and Cx43 **(C)**. In (A) and (B), lanes are as follows: 1, day 5; 2, day 10; 3, day 15 of the estrous cycle. Standards are indicated by their mol-wt values ($\times 10^3$) in (A) and (B). In (C), densitometry is expressed as arbitrary optical density units. For Cx43, ^{a,b,c} values differ ($p < 0.05$).

Table 2
Number of Ovarian Follicles Analyzed for the Presence of Cx26, Cx32, and Cx43

	Day of the estrous cycle				
Type of follicles	13 (7)[a]	14 (4)	15 (6)	16 (7)	Total number of follicles
Preantral					
Primordial	>100	>100	>100	>100	>400
Primary and secondary	13	7	4	9	33
Antral-healthy					
Small	21	24	29	19	93
Medium	6	3	8	3	20
Large	4	5	7	5	21
Antral-atretic					
Small	10	11	5	16	42
Medium	9	6	2	4	21
Large	2	2	1	4	9

[a]Numbers in parenthesis indicate number of ewes.

plished by using a biotinylated secondary antibody (goat antirabbit IgG; Zymed) and the ABC system (Vectastatin; Vector Labs., Burlingame, CA). The color reaction was developed by incubating sections for 5 min with the peroxidase substrate (for Cx26 and Cx32, SG [Vector Labs.]; and for Cx43 3,3'-diaminobenzidine [Vector Labs.]). Control staining consisted of replacing the primary antibody with the same dilution of rabbit serum. Adjacent (serial) sections were used for primary antibody and control staining.

For immunofluorescent localization of Cx43, luteal tissue sections were treated as described above, but detection of primary antibody was accomplished by using FITC-conjugated secondary antibody (goat antirabbit IgG; Boehringer Mannheim, Indianapolis, IN). Control staining consisted of replacing the primary antibody with the same dilution of rabbit serum *(2,14)*.

To evaluate specificity of staining for Cx26, Cx32, and Cx43, antibodies (Zymed) were preabsorbed by using Cx26, Cx32, or Cx43 peptides (Zymed). Antibodies were mixed with the appropriate peptide at the ratio of antibody:peptide recommended by the manufacturer, and incubated for 3 h at room temperature. The ovarian sections were treated as described above, but the primary antibody was replaced by the mixture of preincubated antibody:peptide.

Image Analysis

For luteal tissues, the percentage of the total area that exhibited immunofluorescent staining for Cx43 was evaluated quantitatively with an image analysis system (VIDAS ver. 2.5; Roche Image Analysis System, Los Altos, CA) as described previously *(14)*. For each CL, seven randomly chosen fields (0.025 mm^2/field) were evaluated in each of two tissue sections ($n = 14$ measurements/CL). Background fluorescence was minimal and was adjusted to the same level for each section by the image analysis system. The data are reported as the mean percentage ± SEM of the total area within each field that exhibited positive staining.

Western Immunoblot Analysis

Western analysis was performed as described before in detail *(14)*. Briefly, a portion of CL from days 5, 10, and 15 ($n = 3$/d) was homogenized and then sonicated. Samples of protein (100 µg) from luteal tissues or mouse liver lysate (Zymed; used as a control for Cx26 and Cx32 immunoblot) or rat heart (30 µg, used as a control tissue for Cx43 immunoblot) were added to loading buffer, boiled for 2 min, and then applied to a 12% polyacrylamide gel with a 3% stacking gel *(70)*. After electrophoresis, separated proteins were electroblotted onto an Immobilon-P membrane (Milipore, Bedford, MA), and then immunoblotted with a polyclonal or MAb against Cx26, an MAb against Cx32, or a polyclonal antibody against Cx43 (Zymed). Membranes then were incubated with a peroxidase-labeled antirabbit or antimouse antibody (Amersham International plc, Little Chalfont, UK) followed by detection with ECL reagents (Amersham). Densitometry was performed by using a scanning densitometer (Model PDSI; Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Data for area of positive staining for Cx43 in luteal tissues and for densitometry of Western immunoblots were analyzed by using the general linear models (GLM) analysis of variance, with the main effect of day of the estrous cycle *(71)*. When an F-test was significant ($p < 0.05$), differences between specific means were evaluated by Bonferroni's multiple-comparison procedure *(72)*.

Acknowledgments

We acknowledge Dr. M. L. Johnson, J. D. Kirsch, and K. C. Kraft for expert technical assistance, and J. Berg for

typing the manuscript. We also thank E. M. Hendrix (Department of Biomed. Science, Southwest Missouri State University of Springfield) and W. J. Larsen (Department of Anatomy and Cell Biology, University of Cincinnati) for their gift of antibody against Cx43. This work was supported by NIH grant 1R29 HD30348 to A. T. G.-B. and by USDA grant 93-37203-9721 to D. A. R. and L. P. R.

References

1. Hirshfield, A. N. (1991). *Int. Rec. Cytol.* **124,** 43–101.
2. Jablonka-Shariff, A., Grazul-Bilska, A. T., Redmer, D. A., and Reynolds, L. P. (1993). *Endocrinology* **133,** 1871–1879.
3. Jablonka-Shariff, A., Fricke, P. M., Grazul-Bilska, A. T., Reynolds, L. P., and Redmer, D. A. (1994). *Biol. Reprod.* **51,** 531–540.
4. Reynolds, L. P., Grazul-Bilska, A. T., Killilea, S. D., and Redmer, D. A. (1994). *Prog. Growth Factor Res.* **5,** 159–175.
5. Grazul-Bilska, A. T., Reynolds, L. P., Jablonka-Shariff, A., and Redmer, D. A. (1994). *Assisted Reprod. Technol./Androl.* **6,** 264–286.
6. Smith, M. F., McIntush, E. W., and Smith, G. W. (1994). *J. Anim. Sci.* **72,** 1857–1872.
7. Redmer, D. A. and Reynolds, L. P. (1996). *Rev. Reprod.* **1,** 182–192.
8. Grazul-Bilska, A. T., Redmer, D. A., and Reynolds, L. P. (1997). *Seminar Reprod. Endocrinol.* **15,** 383–393.
9. Holder, J. W., Elmore, E., and Barrett, J. C. (1993). *Cancer Res.* **53,** 3475–3485.
10. Bruzzone, R., White, T. W., and Paul, D. L. (1996). *Eur. J. Biochem.* **238,** 1–27.
11. Goodenough, D. A., Goliger, J. A., and Paul, D. L. (1996). *Annu. Rev. Biochem.* **65,** 475–502.
12. Kumar, N. M. and Gilula, N. B. (1996). *Cell* **84,** 381–388.
13. Yamasaki, H. and Naus, C. C. G. (1996). *Carcinogenesis* **17,** 1199–1213.
14. Grazul-Bilska, A. T., Redmer, D. A., Johnson, M. L., Jablonka-Shariff, A., Bilski, J. J., and Reynolds, L. P. (1996). *Biol. Reprod.* **54,** 1279–1287.
15. Grazul-Bilska, A. T., Reynolds, L. P., and Redmer, D. A. (1997). *Biol. Reprod.* **57,** 947–957.
16. Redmer, D. A., Grazul-Bilska, A. T., and Reynolds, L. P. (1991). *Endocrinology* **129,** 2757–2766.
17. Loewenstein, W. R. (1979). *Biochim. Biophys. Acta.* **560,** 1–65.
18. Risek, B., Klier, F. G., and Gilula, N. B. (1994). *Dev. Biol.* **164,** 183–196.
19. Khan-Dawood, F. S., Yang, J., and Dawood, Y. (1996). *J. Clin. Endocrinol. Metab.* **81,** 835–842.
20. Vander Molen, M. A., Rubin, C. T., McLeaod, K. J., McCauley, L. K., and Donahue, H. J. (1996). *J. Biol. Chem.* **271,** 12,165–12,171.
21. Wiesen, J. F. and Midgley, A. R. (1993). *Endocrinology* **133,** 741–746.
22. Philips, D. M. and Dekel, N. (1991). Mol. Reprod. Dev. **28,** 297–306.
23. Valdimarasson, G., De Sousa, P. A., and Kidder, G. M. (1993). *Mol. Reprod. Dev.* **36,** 7–15.
24. Beyer, E. C., Paul, D. L., and Goodenough, D. A. (1990). *J. Membr. Biol.* **116,** 187–194.
25. Risek, B., Guthrie, S., Kumar, N., and Gilula, N. B. (1990). *J. Cell. Biol.* **110,** 269–282.
26. Risek, B., Klier, F. G., and Gilula, N. B. (1995). *J. Cell Sci.* **108,** 1017–1032.
27. Koike, K., Watanabe, H., Hiroi, M., and Tonosaki, A. (1993). *J. Electron Microsc.* **42,** 94–106.
28. Sutovsky, P., Flechon, J. E., Flechon, B., Motlik, J., Peynot, N., Chesne, P., et al. (1993). *Biol. Reprod.* **49,** 1277–1287.
29. Wiesen, J. F. and Midgley, A. R. (1994). *Biol. Reprod.* **50,** 336–348.
30. Mayerhofer, A. and Garfield, R. E. (1995). *Mol. Reprod. Dev.* **41,** 331–338.
31. Itahana, K., Morikazu, Y., and Takeya, T. (1996). *Endocrinology* **137,** 5036–5044.
32. Okuma, A., Kuraoka, A., Lida, H., Inai, T., Wasano, K., and Shibata, Y. (1996). *J. Reprod. Fertil.* **107,** 255–264.
33. Johnson, M. L., Reynolds, L. P., Redmer, D. A., and Grazul-Bilska, A. T. (1996). *Biol. Reprod.* **54 (Suppl. 1),** 156 (abstract 397).
34. Johnson, M. L., Reynolds, L. P., Redmer, D. A., and Grazul-Bilska, A. T. (1997). In: *Program of the 79th Annual Meeting of the Endocrine Society*, Minneapolis, MN. Program and Abstracts: The Endocrine Society Press. Bethesda, MD, p. 527 (abstract P3-363).
35. Johnson, M. L., Reynolds, L. P., Redmer, D. A., and Grazul-Bilska, A. T. (1997). *Biol. Reprod.* **56 (Suppl. 1),** 125 (abstract 172).
36. Jablonka-Shariff, A., Reynolds, L. P., and Redmer, D. A. (1996). *Biol. Reprod.* **55,** 693–702.
37. Lenhart, J. A., Downey, B. R., and Bagnell, C. A. (1998). *Biol. Reprod.* **58,** 583–590.
38. Itahana, K., Tanaka, T., Morikazu, Y., Komatu, S., Ishida, N., and Takeya, T. (1998). *Endocrinology* **139,** 320–329.
39. Perez-Armendariz, E. M., Luna, J., Aceves, C., and Tapia, D. (1995). *Dev. Growth Differ.* **37,** 421–431.
40. Rhee, S. K., Bevans, C. G., and Harris, A. L. (1996). *Biochemistry* **35,** 9212–9223.
41. Sosinsky, G. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 9210–9214.
42. Hendrix, E. M., Mao, S. J. T., Everson, W., and Larsen, W. J. (1992). *Mol. Reprod. Dev.* **33,** 27–38.
43. Saez, J. C., Berthoud, V. M., Moreno, A. P., and Spray, D. C. (1993). In: *Advances in Second Messenger and Phosphoprotein Research*. Shenolikar, S. and Nairn, A. C. (eds.). Raven, New York, pp. 163–198.
44. Grazul-Bilska, A. T., Reynolds, L. P., Kirsch, J. D., and Redmer, D. A. (1996). *Biol. Reprod.* **54,** 538–545.
45. Grazul-Bilska, A. T., Redmer, D. A., and Reynolds, L. P. (1996). *Endocrine* **5,** 225–233.
46. Grazul-Bilska, A. T., Reynolds, L. P., Kirsch, J. D., Bilski, J. J., and Redmer, D. A. (1996). *Prostaglandins* **52,** 285–302.
47. Moor, R. M., Smith, M. W., and Dawson, R. M. C. (1980). *Exp. Cell Res.* **126,** 15–29.
48. Stagg, R. B. and Fletcher, W. H. (1990). *Endocr. Rev.* **11,** 302–325.
49. Godwin, A. J., Green, L. M., Walsh, M. P., McDonald, J. R., Walsh, D. A., and Fletcher, W. H. (1993). *Mol. Cell Biochem.* **127/128,** 293–307.
50. Albertini, D. F. and Anderson, E. (1975). *Anat. Rec.* **181,** 171–194.
51. Burghardt, R. C. and Matheson, R. L. (1982). *Dev. Biol.* **94,** 206–215.
52. Zheng, J., Fricke, P. M., Reynolds, L. P., and Redmer, D. A. (1994). *Biol. Reprod.* **51,** 623–632.
53. Allen, F. (1987). *Sci. Prog. Oxford* **71,** 275–293.
54. Cameron, I. L. (1971). In: *Cellular and Molecular Renewal in the Mammalian Body.* Cameron, I. L. and Thrasher, J. D. (eds.) Raven, New York, pp. 45–85.
55. Warner, A. (1992). *Semin. Cell Biol.* **3,** 81–91.
56. Girsh, E., Greber, Y., and Meidan, R. (1995). *Biol. Reprod.* **52,** 954–962.
57. Pate, J. L. (1996). *Theriogenology* **45,** 1381–1398.
58. Grazul-Bilska, A. T., Redmer, D. A., and Reynolds, L. P. (1991). *J. Anim. Sci.* **69,** 2099–2107.
59. Hansel, W., Alila, H. A., Dowd, J. P., and Milvae, R. A. (1991). *J. Reprod. Fertil.* **43 (Suppl.),** 77–89.

60. Niswender, G. D. and Nett, T. M. (1994). *In: The Physiology of Reproduction*, Knobil, E. and Neil, J. (eds.). Raven, New York, pp. 781–816.
61. Hansel, W. and Blair, R. M. (1996). *Theriogenology* **45,** 1267–1284.
62. Milvae, R. A., Hinckley, S. T., and Carlson, J. C. (1996). *Theriogenology* **54,** 1327–1349.
63. Simionescu, M., Simionescu, N., Renkin, E. M., and Michael, C. C. (1984). In: *Handbook of Physiology, section 2: The Cardiovascular System, vol. IV Microcirculation, part 1,* Renkin, E. M. and Michel, C. C. (eds.). Waverly, Baltimore, pp. 41–101.
64. Dejana, E., Corada, M., and Lampugnani, A. G. (1995). *FASEB J.* **9,** 910–918.
65. Nelles, E., Butzler, C., Jung, D., Temme, A., Gabriel, H.-D., Dahl, U., et al. (1996). *Proc. Natl. Acad. Sci. USA* **93,** 9565–9570.
66. Reaume, A. G., de Sousa, P. A., Kulkarni, S., Langille, B. L., Zhu, D., Davies, T. C., et al. (1995). *Science* **267,** 1831–1843.
67. Simon, A. M., Goodenough, D. A., Li, E., and Paul, D. L. (1997). *Nature* **385,** 527–528.
68. Oyoyo, U. A., Shah, U. S., and Murray, S. A. (1997). *Endocrinology* **138,** 5385–5397.
69. Grazul-Bilska, A. T., Bilski, J. J., Giles, H., Redmer, D. A., and Reynolds, L. P. (1998). *Biol. Reprod.* **59 (Suppl. 1),** in press.
70. Laemmli, E. K. (1970). *Nature* **227,** 680–685.
71. *SAS User's Guide* (1985). *Statistics*, 5th ed., Statistical Analysis System Institute, Cary, NC.
72. Kirk, R. E. (1982). *Experimental Design*: *Procedures for the Behavioral Sciences*, 2nd ed. Brooks/Cole, Belmont, CA.