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Gap Junctional Intercellular Communication of Bovine Granulosa and Thecal Cells from Antral Follicles: Effects of Luteinizing Hormone and Follicle-Stimulating Hormone

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Throughout each estrous cycle, the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are involved in regulation of folliculogenesis. We have shown that LH or FSH affect cellular interactions mediated by gap junctions in bovine granulosa and thecal cells in vitro. To evaluate further the hypothesis that gonadotropins influence gap junctional intercellular communication (GJIC) and expression of gap junctional proteins known as connexins (Cx), throughout antral follicle development, granulosa and thecal cells from large (>10 mm; n = 13), medium (5–10 mm; n = 20), and small (<5 mm; n = 27) follicles were cultured (n = 4 cultures per size) with or without LH, FSH, or LH + FSH for 24 h. GJIC was evaluated (n = 125–150 cells/treatment group) by using the fluorescent recovery after photobleaching technique and laser cytometry. Additionally, Cx43, Cx32, and Cx26 were detected in cultured cells by immunocytochemistry and Cx43 by Western immunoblot analysis. Finally, progesterone production by cultured cells was evaluated by radioimmunoassay. Across all follicles and treatments, GJIC was greater ($p < 0.01$) for granulosa than thecal cells (4.9 ± 0.05 vs 3.8 ± 0.04%/min). For granulosa cells of large and medium follicles, LH and/or FSH did not affect GJIC. For granulosa cells of small follicles, FSH increased ($p < 0.05$), but LH or LH + FSH had no effect on GJIC. For thecal cells of large follicles, LH increased ($p < 0.01$) GJIC, whereas FSH or LH + FSH had no effects. For thecal cells of medium and small follicles, LH and/or FSH did not affect GJIC. These results demonstrate that FSH influenced GJIC of granulosa cells from small, but not from medium or large, follicles, and LH influenced GJIC of thecal cells from large, but not from medium or small, follicles. Cx43 was present as punctate staining between granulosa or thecal cells from all cultures, indicating assembled gap junctions. LH + FSH increased ($p < 0.05$) expression of Cx43 only by thecal cells from large follicles. Cx32 was detected in the perinuclear cytoplasm of cultured granulosa or thecal cells, and in the cytoskeleton of a few cells per culture dish in all sizes of follicles. Cx26 was present in a regular pattern throughout the cytoplasm of granulosa or thecal cells in all sizes of follicles. For granulosa cells from large follicles, progesterone production was stimulated ($p < 0.05$) with LH or FSH alone but was unaffected by LH + FSH. For granulosa cells from medium and small follicles, progesterone production was unaffected by LH and/or FSH. For thecal cells from all sizes of follicles, LH, FSH, and LH + FSH stimulated ($p < 0.05$) production of progesterone. These data indicate that LH and FSH influence gap junction function and expression, which likely contributes to the development and maintenance of ovarian follicles.

Key Words: Connexin; cow; ovary; follicle; luteinizing hormone; follicle-stimulating hormone.

Introduction

Follicular growth in mammals is a developmentally regulated process that has been well characterized *(1)*. Mature follicles contain a germ cell (oocyte) as well as somatic cells that are steroidogenic (parenchymal: granulosa and thecal cells) or nonsteroidogenic (nonparenchymal: fibroblasts, endothelial cells, smooth muscle cells, and others). The primordial follicle consists of only a few cells, but these develop into a preovulatory follicle containing thousands of highly differentiated and diverse cell types. This developmental process is strictly regulated; only a few of the many follicles that begin development will progress to the preovulatory stage *(1)*. Most will regress through the degenerative process of atresia *(1)*. The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), influence whether antral follicles will develop until ovulation or become atretic *(2)*. FSH and LH are involved in regulation of the growth,

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Table 1
Effects of LH and FSH on GJIC for Granulosa and Thecal Cells Isolated from Large, Medium, and Small Follicles

		Treatment[a]			
Follicle size	Cell type	Control	LH	FSH	LH + FSH
Large	Granulosa	4.8 ± 0.2 (138)	4.6 ± 0.2 (125)	5.0 ± 0.2 (146)	4.8 ± 0.2 (144)
	Thecal	3.3 ± 0.1 (139)	3.9 ± 0.1[b] (147)	3.7 ± 0.1 (141)	3.8 ± 0.1 (137)
Medium	Granulosa	4.7 ± 0.2 (131)	4.9 ± 0.2 (146)	4.9 ± 0.2 (144)	4.6 ± 0.2 (139)
	Thecal	4.0 ± 0.1 (143)	4.0 ± 0.1 (145)	4.0 ± 0.1 (143)	3.6 ± 0.1 (144)
Small	Granulosa	4.9 ± 0.2 (150)	4.6 ± 0.2 (126)	5.4 ± 0.2[c] (138)	5.2 ± 0.2 (141)
	Thecal	3.6 ± 0.2 (137)	3.9 ± 0.1 (143)	3.9 ± 0.2 (139)	4.0 ± 0.2 (145)

[a] Data (mean ± SEM) are presented as the initial rate of fluorescence recovery (percentage of the prebleach value recovered per minute) calculated during the first 4 min after photobleaching. Numbers in parentheses indicate the number of cells evaluated.
[b] $p < 0.01$. Means differ from control (no treatment) within each row.
[c] $p < 0.05$. Means differ from control (no treatment) within each row.

development, and maturation of antral follicles *(1,3,4)*. FSH induces antrum formation and stimulates production of LH receptors in granulosa cells *(5)*. FSH and/or LH appear to be involved in both maintenance of cell proliferation and prevention of atresia in ovarian follicles *(4,6,7)*. In addition, FSH and/or LH have major roles in promoting steroid production by ovarian follicles *(1,5,8)*.

Cell-to-cell communication through specialized channels known as gap junctions is critical for coordinating cell function during normal tissue growth *(9,10)*. The possibility that gap junctions have a functional role in follicular development was suggested more than two decades ago *(11,12)*. We and others have shown that gap junctional proteins connexin 43 (Cx43) and Cx26 are present in healthy growing bovine follicles across all stages of follicular development and that their pattern of expression suggests that they may have a role in maintaining a healthy follicle *(13,14)*. The role of connexins in regulation of follicular growth and atresia has also been suggested for other species *(15–26)*. Lack of Cx43 has been reported to affect follicular development *(27,28)*.

We previously demonstrated that gap junction expression changes during follicular development and suggested that the changes were based on hormonal and/or other factors *(13,21,29)*. However, exactly how hormones interact with gap junctions during follicular growth and death is still relatively undefined *(1,15,30)*. Gonadotropins have been reported to influence gap junction formation and function in reproductive organs, including the ovary *(19,20,22)*. The role of human chorionic gonadotropin (hCG), LH, and FSH in regulation of expression of gap junctions and gap junctional intercellular communication (GJIC) have been demonstrated in several species and in a variety of cell types, including granulosa cells *(19,20)*. The aim of the present in vitro study was to evaluate the effects of FSH and/or LH on the expression and function of gap junctions in bovine granulosa and thecal cells during follicular development.

Results

Effects of LH and/or FSH on GJIC

Granulosa and thecal cells exhibited GJIC in all cell cultures. Across all follicles and treatments, the GJIC during the first 4 min after photobleaching was greater ($p < 0.01$) for granulosa than thecal cells (4.9 ± 0.05 vs 3.8 ± 0.04%/min, respectively).

For granulosa cells of large and medium follicles, LH and/or FSH did not affect GJIC. For granulosa cells of small follicles, FSH increased ($p < 0.05$) GJIC, but LH or LH + FSH had no effects on GJIC (Table 1).

For thecal cells of large follicles, LH increased ($p < 0.01$) GJIC, whereas FSH alone or LH + FSH had no effects on GJIC. For thecal cells of medium and small follicles, LH and/or FSH did not affect GJIC (Table 1).

For all cells cultured, cells that were not photobleached (positive control) or cells that were photobleached and not in contact with other cells (negative control) had no change in GJIC, indicating that nonspecific photobleaching or leakage of fluorescent probe from the cells did not occur.

Immunocytochemical Localization of Cx Proteins

Staining patterns for the Cx proteins in granulosa and thecal cells were similar across all cultures, sizes of follicles, and treatments. Cx43 was present on the cellular borders and occasionally in the cytoplasm or cellular membrane of granulosa cells (Fig. 1A) or thecal cells (Fig. 1B) from all cultures, demonstrating that assembled gap junctions were present. Cx32 staining was detected in granulosa (Fig. 1C) and thecal cell cultures (Fig. 1D) in the perinuclear area of the cytoplasm of the majority of cells, or, in a few cells, the staining was associated with the cytoskeleton of the cells. Cx26 was present in a regular pattern throughout the cytoplasm and also at the cellular borders of granulosa (Fig. 1E) or thecal cells (Fig. 1F).

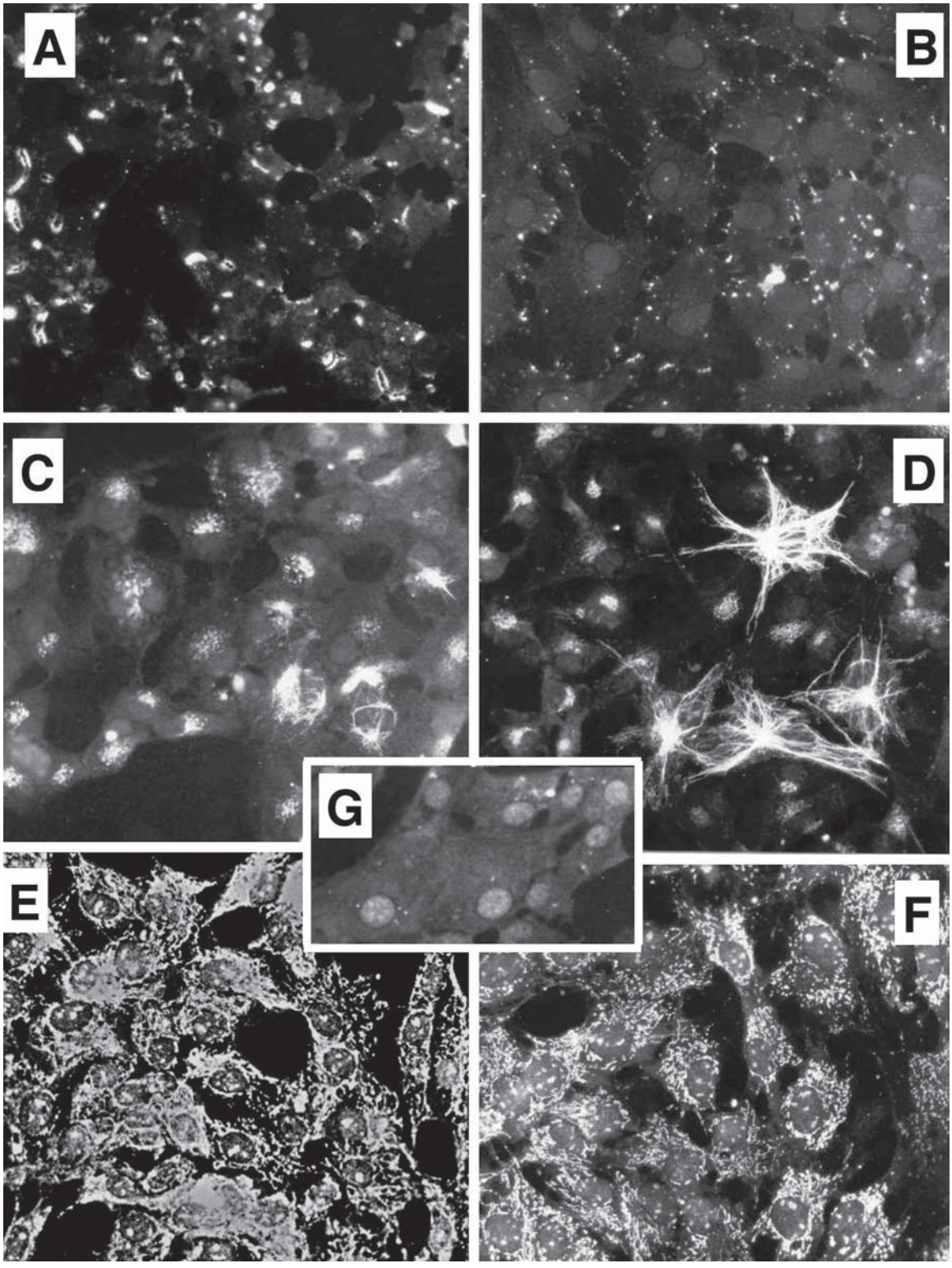

Fig. 1. Immunolocalization of (**A,B**) Cx43, (**C,D**) Cx32, and (**E,F**) Cx26 in cultured granulosa cells (**A,C,E**) or thecal cells (**B,D,F**). Note the punctate appearance of Cx43 staining at the periphery of the cells (**A,B**) compared with Cx26 staining, which is present throughout the cytoplasm (**E,F**). In (**C**) and (**D**) the presence of Cx32 is shown in the perinuclear area of most cells and in the cytoskeleton of only a few cells. (**G**) Control staining.

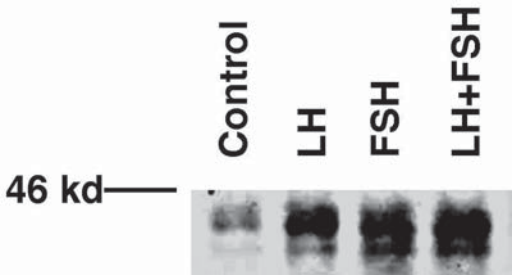

Fig. 2. Representative Western immunoblot analysis of Cx43 in bovine thecal cells isolated from a large (>10 mm) follicle treated with or without (Control) LH, FSH, or LH + FSH. Note the presence of three bands for Cx43, representing phosphorylated forms of the protein. The standard for molecular weight value ($\times 10^{-3}$) is indicated on the left.

Table 2
Effects of LH and FSH on Expression of Cx43 Protein by Granulosa and Thecal Cells Isolated from Large, Medium, and Small Follicles

		Treatment[a]			
Follicle size	Cell type	Control	LH	FSH	LH + FSH
Large	Granulosa	1.00	2.99 ± 2.76	2.58 ± 2.34	0.34 ± 0.09
	Thecal	1.00	3.21 ± 0.07	3.29 ± 0.80	6.53 ± 2.41[b]
Medium	Granulosa	1.00	2.77 ± 1.23	3.47 ± 2.33	2.19 ± 1.51
	Thecal	1.00	1.41 ± 0.23	1.03 ± 0.30	1.18 ± 0.04
Small	Granulosa	1.00	1.40 ± 0.77	5.45 ± 4.62	8.29 ± 7.44
	Thecal	1.00	1.55 ± 0.19	2.10 ± 1.40	3.94 ± 3.32

[a] Values (mean ± SEM) are expressed in fold increase or decrease in arbitrary densitometric units with the control as 1.00.

[b] $p < 0.05$. Means differ from control (no treatment) within each row.

Effects of LH and/or FSH on Expression of Cx43 Protein

Western immunoblot analysis of Cx43 revealed that Cx43 protein was present as three bands with an M_r between 43 and 46 kDa (Fig. 2). The presence of three bands is indicative of a difference in phosphorylation of the Cx protein.

For granulosa cells, because of the variability among assays, the effects of treatments with LH, FSH, and LH + FSH on expression of Cx43 protein were similar across all sizes of follicles (Table 2). However, the following trends were noted: (1) for granulosa from large and medium follicles, treatment with LH or FSH alone appeared to increase Cx43 expression two to threefold; (2) for granulosa from medium follicles, LH + FSH appeared to increase Cx43 expression by twofold; and (3) for granulosa from small follicles, FSH appeared to increase Cx43 expression by five-fold and LH + FSH by eightfold.

For thecal cells, the variability among assays of Cx43 protein expression was less than for granulosa cells (Table 2). For thecal cells isolated from large follicles, LH + FSH increased ($p < 0.05$) expression of Cx43 protein by sixfold compared to no treatment (Table 2). Additionally, the following trends for thecal cells were seen: (1) for thecal cells from large follicles, LH or FSH treatment alone appeared to increase Cx43 expression by threefold; and (2) for thecal cells from small follicles, FSH treatment appeared to increase Cx43 expression by twofold and LH + FSH treatment by fourfold.

Localization of Δ5-3β-Hydroxysteroid Dehydrogenase (3β-HSD)

Staining of granulosa and thecal cells revealed that the majority of the cells were steroidogenic at the time of evaluation of GJIC and collection of cells for immunocytochemistry (ICC), Western immunoblot analysis, and evaluation of progesterone production (Fig. 3 A–C).

Effects of LH and/or FSH on Progesterone Production

The effects of LH and FSH on progesterone production were compared as a fold increase value (Table 3), with the control value (no LH or FSH treatment) set to 1.0. For granulosa cells of medium and small follicles, LH and FSH, alone or in combination, had no effect on progesterone production. For granulosa cells of large follicles, LH or FSH alone stimulated ($p < 0.05$) progesterone production, but progesterone production was not affected when cells were treated with LH + FSH (Table 3). For thecal cells, across all sizes of follicles, LH, FSH, and LH + FSH stimulated ($p < 0.05$) production of progesterone (Table 3).

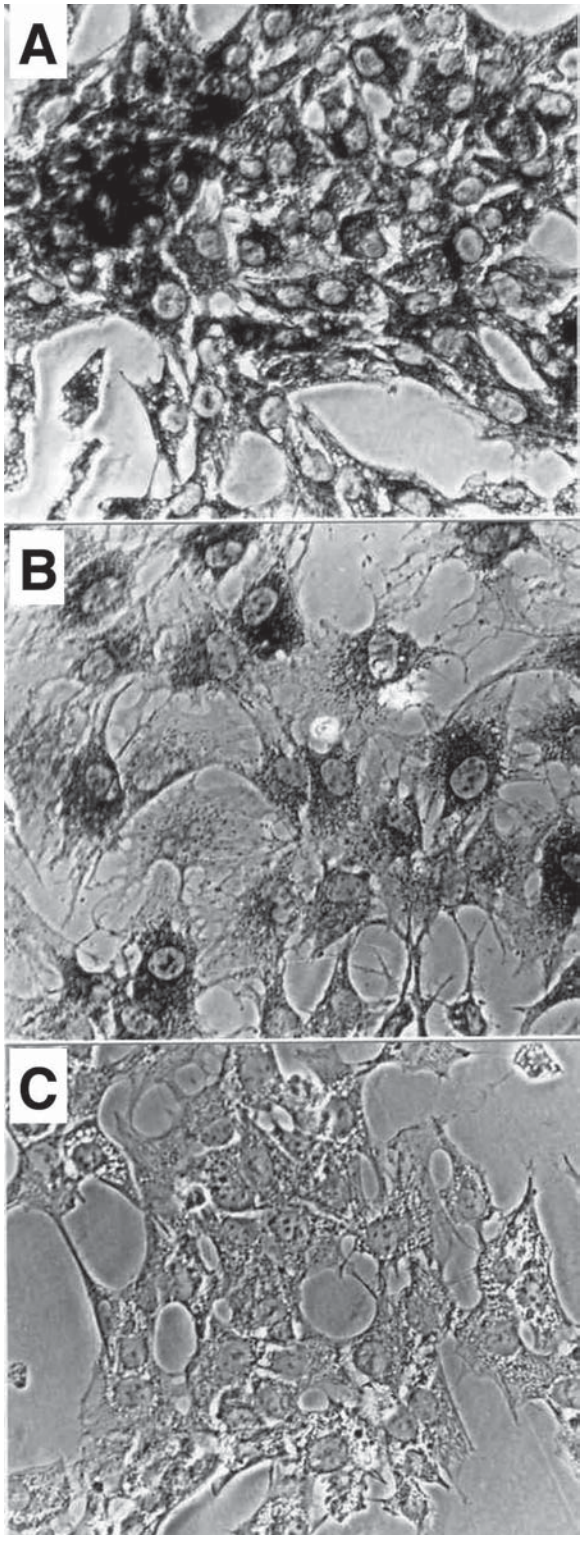

Fig. 3. Localization of 3β-HSD in (**A**) granulosa and (**B**) thecal cells. (**C**) Control staining for 3β-HSD. The presence of 3β-HSD in cultured cells is shown by formazan granules (dark areas) in the cytoplasm.

Discussion

Granulosa cells or thecal cells were isolated from antral follicles of different size (corresponding to the various stages of antral follicular development) so that the effects of gonadotropins on gap junctional function and expression of connexins could be compared during follicular development. The involvement of FSH and LH in regulation of the growth, development, and maturation of antral follicles has been shown to be stage specific *(1,3,4)*. The present data demonstrated that the effects of LH and/or FSH effects on GJIC, expression of connexins, and progesterone production were stage specific and were different in granulosa cells compared with thecal cells.

The differences in function and the position occupied by granulosa cells vs thecal cells in the follicle may be responsible for our data showing that, across all treatments, granulosa cells had a greater GJIC than thecal cells. The granulosa layer is avascular; therefore, cellular signaling is mediated by paracrine and gap junctional pathways. In addition, granulosa cells are directly involved in development of a healthy, mature oocyte and its maintenance in meiotic arrest *(3,31)*. Thecal cells are in a highly vascular environment, surrounded by nonparenchymal cells, and cellular signaling can be mediated by endocrine, paracrine, and gap junctional pathways *(7,19)*. Therefore, GJIC may be more important for granulosa cells than for thecal cells. In addition, we and others have observed greater expression of Cx43 in granulosa than in thecal cells of antral sheep or cow follicles in vivo *(13,14,21)*.

The present data demonstrated that LH or FSH alone influenced GJIC of granulosa and thecal cells in a cell- and stage-specific manner. LH affected GJIC of thecal cells isolated from large follicles, and FSH affected GJIC of granulosa cells isolated from small follicles. The finding that the combination of LH + FSH did not stimulate GJIC in either cell type is puzzling and requires further evaluation. It has been demonstrated that granulosa and thecal cells have different responses to LH and FSH in vivo at varying stages of follicular growth *(2,32)*. In general, LH has its greatest effects on large follicles and particularly on preovulatory follicles while FSH has a major role in promoting follicular growth and maturation during earlier stages of follicular development and expression of LH receptors during the preovulatory period *(3,4,33)*. Our data examining the regulation of GJIC by LH and FSH reflect these complementary roles of the gonadotropins.

We and others have shown that the abundance of Cx43 and gap junctions increased in large antral follicles compared with small or medium follicles, suggesting a role for FSH in promoting gap junction formation and GJIC *(13,14, 18,21)*. Additionally, an in vitro study using a rat granulosa cell line that expressed Cx43 and the FSH receptor has demonstrated that FSH directly stimulated GJIC and increased expression of Cx43 mRNA *(34)*. In vivo, LH and FSH may affect GJIC by stimulating production of second messengers, including cyclic adenosine monophosphate (cAMP), protein kinase C, or calcium, and the effects of the second messengers may depend on the stage of differentiation of the cells *(3,19,20)*. In vitro, contact-dependent interactions were necessary for FSH-stimulated cAMP production by

Table 3
Effects of LH and FSH on Production of Progesterone by Granulosa and Thecal Cells Isolated from Large, Medium, and Small Follicles

		Treatment[a]			
Follicle size	Cell type	Control	LH	FSH	LH + FSH
Large	Granulosa	1.00	1.39 ± 0.08[b]	1.33 ± 0.09[c]	1.27 ± 0.12
	Thecal	1.00	1.57 ± 0.07[b]	1.37 ± 0.09[c]	1.68 ± 0.17[b]
Medium	Granulosa	1.00	1.05 ± 0.14	1.04 ± 0.08	1.21 ± 0.08
	Thecal	1.00	1.70 ± 0.11[b]	1.50 ± 0.06[b]	1.81 ± 0.12[b]
Small	Granulosa	1.00	1.00 ± 0.19	0.97 ± 0.07	1.09 ± 0.11
	Thecal	1.00	1.92 ± 0.19[b]	1.35 ± 0.19[c]	1.90 ± 0.26[b]

[a] Values (mean ± SEM) are expressed in fold increase with control as 1.00. Basal levels of progesterone secreted by granulosa cells were 12.8 ± 1.6, 14.5 ± 0.7, and 19.2 ± 3.4 ng/mL and by thecal cells were 10.8 ± 2.4, 9.2 ± 1.4, and 9.6 ± 2.4 ng/mL for large, medium, and small follicles, respectively.

[b] $p < 0.01$. Means differ from control (no treatment) within each row.

[c] $p < 0.05$. Means differ from control (no treatment) within each row.

rat granulosa cells *(35)*. In human granulosa cells, which had functional gap junctions and expressed Cx43 in vitro, treatment with an analog of cAMP increased the permeability of gap junctions, thus increasing GJIC *(36)*. These data indicate that gonadotropin hormones affect gap junction function and that this action can be mediated by second messengers. However, additional studies should be undertaken to identify the mechanisms of gonadotropin actions on intracellular levels of second messengers in granulosa and thecal cells.

In the present study, Cx43, Cx32, and Cx26 were immunolocalized in both granulosa and thecal cells. It appeared likely that Cx43 was primarily responsible for the GJIC of granulosa and thecal cells because the staining for Cx43 was punctate in appearance and present on the cellular borders of both cell types.

Cx32 and Cx26 were present in the cytoplasm of granulosa and thecal cells, but the appearance of the staining for each Cx was different. Cx32 was present in the perinuclear areas of most cells and was detected in a cytoskeletal array within a few cells. However, in vivo, Cx32 was present only in the cytoplasm of granulosa cells in follicles that were undergoing atresia, and we suggested that this appearance of Cx32 could be a marker for follicular atresia in cows *(13)*. When evaluated for GJIC and expression of connexins, the cultured cells appeared healthy and steroidogenically active, suggesting that the few cultured cells that contained Cx32 protein in a cytoskeleton array might be in the early stage of apoptosis. Dual staining for apoptotic factors and Cx32 should be done in future studies to confirm or refute these findings. Additionally, colocalization of Cx32 with specific cytoskeletal elements (e.g., actin, microtubules, microfilaments) in cultures of granulosa or thecal cells with induced apoptosis should further explain the role of Cx32 in atresia.

The cytoplasmic staining for Cx26 in both granulosa and thecal cells had a more punctate appearance than Cx32 staining, and since it was present along cellular borders as well, Cx26 might also be involved in GJIC in vitro. In vivo, punctate staining for Cx26 was present in both granulosa and thecal cell layers of antral follicles, suggesting that assembled gap junctions were present *(13)*. The relatively great abundance of Cx26 protein in the cytoplasm of cultured cells may suggest that Cx26 has another role in follicular function, e.g., growth control, as recently reported for Cx43 *(37)*. Cytoplasmic staining of Cx32 and Cx26 has been reported for other cell types from other species as well *(20,21,38)*.

In vivo, we have shown that for sheep and cows, expression of Cx43, Cx32, and Cx26 protein and Cx43 mRNA changes during follicular growth and atresia *(13,21,29,39)*. For bovine follicles, Cx43 mRNA and protein and Cx26 protein were present in antral follicles from several stages of follicular development, suggesting a codominant role of these connexins in controlling follicular growth and health *(13,29)*.

The effects of gonadotropins on expression of Cx43 protein have been demonstrated in several species *(19)*. Larsen et al. *(40)* reported that there was a reduction in size of gap junctions at the cell surface after injections of hCG to stimulate ovulation. Recently, several researchers have shown that gonadotropins induced changes both in the amount of Cx43 expressed and in its phosphorylation state in rat and pig ovarian follicles *(17,18,41)*. By ICC, we also observed a tendency for LH and/or FSH to increase Cx43 protein expression in the cultures of bovine granulosa cells and thecal cells (unpublished observation).

In the present study, similar to previous reports, Western immunoblot analysis revealed several bands that apparently represent the different phosphorylation patterns of Cx43 *(13,18,19,21,41,42)*. The phosphorylation pattern of Cx43 and the reasons for changes in phosphorylation are unclear at present. However, there are indications that gonadotropins and other hormones stimulate significant changes in

phosphorylations, which may regulate opening or closing of gap junctional channels *(41–43)*.

In vivo, both granulosa and thecal cells participate in production of estradiol and progesterone, and LH and/or FSH play important roles in regulating the production of steroids *(5,8)*. In the present study, most granulosa and thecal cells stained positively for steroidogenic activity, indicating that the cells were fully functional and differentiated in vitro. Our data demonstrated that progesterone production by granulosa cells from large, but not from medium or small, follicles was stimulated by LH or FSH alone. In addition, progesterone production by thecal cells was stimulated by LH, FSH, and LH + FSH across all sizes of follicles. Other researchers have also evaluated the effects of LH and/or FSH on progesterone production *(44–48)*. Langhout et al. *(44)* have reported that LH or FSH alone had no effect on production of progesterone by cultured granulosa cells. However, Kuran et al. *(46)* demonstrated that in vitro treatments with either LH or FSH promoted differentiation and luteinization of bovine granulosa cells and stimulated production of granulosa cell progesterone in a dose-dependent manner. Recent data have shown that FSH stimulated production of progesterone by bovine granulosa cells from small follicles and that LH increased progesterone production of thecal cells from large follicles *(45,47)*. In vivo, the effects of LH on thecal cells vary with the stage of follicular development, and increased progesterone production is related to increased activity of the C_{27} side-chain cleavage enzyme *(49)*. Thus, these data demonstrate that the effects of LH and FSH on progesterone secretion depend on the cell type, stage of follicular development, and dose of gonadotropin used.

The effects of gonadotropins on granulosa and thecal GJIC and progesterone secretion were different. This suggests that these hormones affect gap junction function and steroid production through separate pathways or mechanisms, and it may indicate that GJIC is not associated with progesterone secretion in vitro. However, the primary purpose of examining progesterone production and determining whether or not the cells were steroidogenic 3β-HSD in our experiments was to confirm that the cells were fully functional in culture when evaluated for GJIC and expression of connexins. Further studies will be needed to evaluate whether there are any relationships between LH or FSH stimulation of GJIC and progesterone secretion for granulosa and thecal cells, as has been reported for luteal cells *(50)*.

In summary, we have shown that the effects of the gonadotropins on gap junction function and expression of connexins by granulosa cells or by thecal cells are unique, causing different responses by granulosa cells than by thecal cells. We have also reported that the effects of each gonadotropin are stage specific, varying with the size of the follicle used for isolation of the granulosa or thecal cells. The presence of Cx43 on the cellular borders and on membranes and not in a cytoplasmic array suggests that Cx43 might be responsible for mediating the effects of LH or FSH on GJIC in vitro. The presence of Cx32 in the cytoskeleton of cells might be associated with apoptotic processes, as was suggested previously *(13)*. Since Cx26 was present on cellular borders as well as in the cytoplasm, Cx26 might cooperate with Cx43 in mediating the effects of LH or FSH on GJIC in vitro and have additional effects (e.g., control of cell proliferation). Finally, LH or FSH had specific effects on progesterone productions by either cell type.

Data from in vitro experiments, such as the one reported here, may help us understand what happens in vivo during follicular development. Ultimately, these studies may contribute to a better understanding of how follicular growth is regulated during normal and abnormal conditions.

Materials and Methods

Reagents

For cell cultures, Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium, Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, fetal bovine serum (FBS), calf serum, crystalline bovine insulin, trypan blue stain (0.4%), and penicillin-streptomycin (P/S) (10,000 U of penicillin G sodium salt and 10,000 μg of streptomycin sulfate/mL) were purchased from Gibco (Grand Island, NY). Bovine serum albumin (BSA) (fractionV), dimethylsulfoxide, transferrin, heparin, hydrocortisone, etiocholan-3β-ol-17-one, nitroblue tetrazolium, EDTA, EGTA, dithiothretol (DTT), phenylmethylsulfonyl fluoride (PMSF), 4-(2-aminoethyl)-benezene-sulfonylfluoride (AEBSF), leupeptin, pepstatin, *N*-α-p-tosyl-L-lysine (TLCK), and *N*-α-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma (St. Louis, MO). LH (USDA bovine LH b5) and FSH (USDA bovine FSH b1) were obtained from the USDA Animal Hormone Program and the National Hormone and Pituitary Program, Beltsville, MD. The fluorescent probe, calcein-AM, was purchased from Molecular Probes (Eugene, OR). For radioimmunoassay (RIA), progesterone standard was purchased from Sigma, and tritiated progesterone from DuPont/New England Nuclear (Boston, MA). The progesterone antibody (GDN 337) was kindly supplied by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO). Rabbit polyclonal antibodies against Cx43, Cx32, and Cx26 were purchased from Zymed (San Francisco, CA). Goat anti–rabbit IgG conjugated to fluorescein isothiocyanate (FITC) and normal rabbit serum were purchased from Boehringer Mannheim (Indianapolis, IN). Electrochemiluminescence reagents and anti–rabbit IgG conjugated to peroxidase were purchased from Amersham (Piscataway, NJ).

Tissue Collection

Ovaries ($n = 37$ from 35 cows) from the mid- to late stages of the estrous cycle (evaluated as previously described; *[51]*) were obtained from a local slaughterhouse and transported to the laboratory in ice-cold phosphate-buffered saline (PBS)

containing 2% P/S. Before dissection, antral follicles were measured and classified as small (<5 mm; $n = 27$), medium (5–10 mm; $n = 20$), and large (>10 mm; $n = 13$) antral follicles. Follicles that appeared healthy (e.g., were vascularized and had transparent follicular fluid) were used for cell collection.

Culture of Granulosa and Thecal Cells

Granulosa and thecal cells were isolated for culture using techniques previously described *(52)*. After removal of follicular fluid, granulosa cells were removed from the antrum by trituration with DMEM containing heparin (100 USP units/mL) and separated from medium or follicular fluid by centrifugation. The thecal layer was dissected from the antrum and thecal cells were dissociated by using collagenase. After dissociation, cells were counted and the viability of cells was assayed using trypan blue dye exclusion *(53)*. To obtain an adequate number of viable cells for evaluation of GJIC and all other evaluations described subsequently, granulosa or thecal cells were pooled from small ($n = 6–7$ follicles/culture), medium ($n = 4–7$ follicles/culture), or large ($n = 3–4$ follicles/culture) follicles. Cells were resuspended in plating medium (DMEM containing 2% serum [1% FBS and 1% calf serum] and P/S), plated as described later, and preincubated in 35- or 60-mm Petri dishes for 48 h at 37ºC in a humidified atmosphere (5% CO_2 and 95% air). For evaluation of GJIC, ICC, and 3β-HSD detection, granulosa cells were plated into the center of a 35-mm dish at a concentration of 5×10^4 cells/dish and thecal cells at 2.5×10^4 cells/dish. For Western immunoblot analysis, granulosa or thecal cells were plated into two 60-mm dishes at a concentration of $0.5\text{-}1 \times 10^6$ cells/dish.

After preincubation, the plating medium was removed and cells were incubated for an additional 24 h in serum-free medium *(53)* containing no treatment (control), LH (30 ng/mL), or FSH (100 ng/mL), or LH + FSH (30 and 100 ng/mL, respectively) followed by evaluation of GJIC, expression of connexins (by ICC and/or Western immunoblot analysis), and progesterone concentration in culture medium. The doses of LH and FSH were selected based on a preliminary dose response experiment *(39)*. Treatments were performed and evaluated in duplicate for each of the four cultures of each follicle size.

Evaluation of GJIC

After incubations with treatments, medium in each dish of cells was replaced with serum-free medium containing the fluorescent probe calcein-AM (20 μ*M*) and incubated for 10 min at room temperature (24 to 25ºC). Then, cells were washed to completely remove excess calcein-AM, and the dishes were placed onto the interactive laser cytometer. Three fields of cells were identified for scanning for each dish. Within each field, 8–12 cells were selected and analyzed for initial fluorescence intensities. The fluorescent probe was then photobleached in four to six of the selected cells as previously described *(53)*. The fluorescent recovery after photobleaching was evaluated using the linear portion of the recovery curve (the first 4 min after photobleaching) also as previously described *(53)*.

ICC for Cx43, Cx32, and Cx26 in Cultured Granulosa and Thecal Cells

ICC for Cx43, Cx32, and Cx26 in cultured granulosa and thecal cells was done as reported previously *(21,54)*. Cultured cells were fixed in ethanol:glacial acetic acid (5.7:1) for 20 min and rinsed several times in PBS containing Triton X-100 (0.3% [v/v]). Fixed cells were treated for 20 min with blocking buffer (PBS + 0.3% Triton X-100 + normal goat serum (1 to 2% [v/v]) and then incubated overnight at 4ºC with rabbit polyclonal antibody against Cx43, Cx32, and Cx26. Primary antibody was detected with goat anti–rabbit IgG conjugated to FITC. Normal rabbit serum was used in place of primary antibody for controls.

Western Immunoblot Analysis of Cx43

After the medium was removed from the dishes, the cells were washed twice in 1 mL/dish of TBS-EDTA (20 m*M* Tris-HCl; 155 m*M* NaCl, pH 7.4), and protein was isolated from the cells as described by Wang and coworkers *(55)*. Cells were incubated in lysis buffer (1 mL/dish) consisting of 2% (w/v) sodium dodecyl sulfate (SDS), 20 m*M* Tris-HCl, 5 m*M* EDTA, 5 m*M* EGTA, 0.5 m*M* PMSF, 10 μg/mL of AEBSF, 5 μg/mL of leupeptin, 10 μg/mL of pepstatin, 10 μg/mL of TLCK, and 10 μg/mL of TPCK at pH 7.4 and allowed to lyse in the dish for 10–15 min at room temperature. The lysate was collected, and the protein was precipitated with 100 μL of 100% trichloroacetic acid (TCA) and centrifuged at 3600*g* for 5 min. The protein pellets were washed with 2.5% (w/v) TCA, recentrifuged, and neutralized with 25 μL of 3 *M* Tris base for 30 min. After adding 25 μL of water to the neutralized pellets, the proteins were homogenized in lysis buffer without SDS containing 1 m*M* DTT and stored frozen (–20ºC) until Western immunoblot analysis was done. Thawed samples were sonicated briefly to solubilize proteins, and protein concentrations were determined by using the Coomassie Brilliant Blue G assay with BSA as the standard *(13,21,54)*.

Samples of protein (40 μg/sample) from cultured cells were separated by SDS polyacrylamide gel electrophoresis and analyzed by Western immunoblot analysis as described previously in detail *(13,21,54)*.

Localization of 3β-HSD

To detect 3β-HSD, a marker for steroidogenic cells, cultured cells were fixed in 10% formalin solution for 15 min and treated with staining solution (PBS containing BSA, nitroblue tetrazolium, steroid [3β-androst-2β-ol-17-one] and NAD^+; *[54]*). Staining solution without steroid was used for controls.

Progesterone RIA

Progesterone concentrations in 100 µL of unextracted culture medium were measured by RIA using methods validated in our laboratory *(52)*. The limit of detection for progesterone was 25 pg/tube.

Statistical Analyses

The effects of treatments and size of follicles on GJIC, progesterone concentrations in medium, and expression of Cx43 protein (analyzed by Western immunoblot analysis) were compared by using the General Linear Models analysis of variance *(56)*. When an F-test was significant ($p < 0.05$), differences between specific means were evaluated by the Dunnett multiple comparison procedure *(57)*.

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