Expression of connexin 43 and gap junctional intercellular communication in the cumulus–oocyte complex in sheep

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Abstract

To evaluate the effects of FSH, LH and/or cAMP on expression of connexin 43 (Cx43) in the ovine cumulus-oocyte complex (COC) and gap junctional intercellular communication (GJIC) of cumulus cells, two experiments were carried out. In experiment 1, Cx43 was immunodetected in the COC, before or after maturation, obtained from non-treated or FSH-treated ewes. The expression of Cx43 in the COC was greater ($P < 0.01$) on day 16 than on day 15 of the estrous cycle. *In vivo* FSH treatment decreased ($P < 0.02$) Cx43 expression on day 16 but not on day 15 of the estrous cycle. In experiment 2, intact COCs or isolated cumulus cells obtained from small and large follicles from FSH-treated ewes were cultured with or without FSH, LH or cAMP agonist and evaluated for GJIC by laser cytometry. For large follicles, the basal rate of GJIC was greater ($P < 0.01$) for cumulus cells in intact COCs than for isolated cumulus cells. FSH increased ($P < 0.04$) GJIC in cumulus cells in intact COCs and tended to increase ($P < 0.1$) GJIC in isolated cumulus cells from small follicles but decreased ($P < 0.01$) GJIC in cumulus cells in intact COCs from large follicles. LH also increased ($P < 0.01$) GJIC in isolated cumulus cells from small follicles but decreased GJIC in intact COCs ($P < 0.01$) and isolated cumulus cells ($P < 0.02$) from large follicles. cAMP increased ($P < 0.01$) the GJIC in both intact COCs and cumulus cells from small and large follicles. These results indicate that day of estrous cycle, stage of maturation and duration of FSH treatment affect expression of Cx43 in ovine COCs. In intact COCs, GJIC in cumulus cells was enhanced, probably due to the presence of the oocyte. In addition, the effects of FSH and LH, but not cAMP, on GJIC of cumulus cells depended on the stage of follicular development and on the presence of the oocyte.

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Introduction

The coordinated function of the two compartments of the follicle, oocyte and granulosa/cumulus cells is mediated by humoral (contact independent) as well as gap junctional (contact dependent) interactions (Eppig 1991, 2001, Grazul-Bilska *et al.* 1997, Granot & Dekel 2002). Gap junctions occur at sites of close cell apposition; they are an array of intercellular membrane channels that allow inorganic ions, second messengers and metabolites (less than about 1000 Da) to pass from cell to cell. Gap junctions are composed of hemi channels (connexons) that consist of six subunit proteins that are termed connexins (Cx; Grazul-Bilska *et al.* 1997). Gap junctions in granulosa/cumulus cells of ovarian follicles may contain several different connexins – including Cx26, Cx32, Cx37, Cx43 and/or Cx45 – depending on the species (Grazul-Bilska *et al.* 1997, Simon *et al.* 1997, Nuttinck *et al.* 2000,

Kidder & Mhawi 2002). Cx43 is one of the major gap junctional proteins expressed in the cumulus oocyte complex (COC) and granulosa cells of several species (Valdimarsson *et al.* 1993, Wiesen & Midgley 1993, Mayerhofer & Garfield 1995, Grazul-Bilska *et al.* 1997, 1998, Johnson *et al.* 1999, Kidder & Mhawi 2002). Models of targeted mutation of Cx43 clearly demonstrated that gap junctional coupling mediated by Cx43 channels plays an indispensable role in both germ line development and postnatal folliculogenesis (Juneja *et al.* 1999).

Normal oocyte growth and differentiation depend on an intimate association between follicular (somatic) cells and the developing germ cells (Eppig 1991). These somatic cell–oocyte interactions via gap junctions are essential for oocyte growth, provision of substrates for energy metabolism, cytoplasmic maturation of the oocyte, inhibition of maternal genes and maintenance of meiotic arrest

(Eppig 1991, Downs 1995, Larsen *et al.* 1996). The oocytes, on the other hand, promote the organization of the follicle, proliferation of granulosa cells, differentiation and function of cumulus cells, and cumulus expansion possibly through gap junctions (Vanderhayden *et al.* 1992, Eppig 2001, Granot & Dekel 2002).

The expression of Cx43 and gap junctional intercellular communication (GJIC) in the ovaries is affected by several factors including: stage of follicular development; hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH) or estradiol; and second messengers (Grazul-Bilska *et al.* 1997, 1998, Johnson *et al.* 1999, 2002, Nuttinck *et al.* 2000, Granot & Dekel 2002, Kolle *et al.* 2003). Several studies have focused on GJIC in the ovarian follicle (Eppig 1991, Downs 1995, Thomas *et al.* 2004), and the role of connexins including Cx43 in folliculogenesis (Granot & Dekel 2002, Kidder & Mhawi 2002). However, expression of Cx43 in the COC, and the effects of oocytes on GJIC in the COC in the presence of gonadotropins or second messengers during oocyte and follicle growth have not been evaluated in detail. Therefore, the current study aimed to: (1) immunolocalize Cx43 in the COC on days 15 and 16 of the estrous cycle in non-treated and FSH-treated ewes and (2) to evaluate the effects of the oocyte on GJIC of cumulus cells in the presence or absence of exogenous gonadotropins or cAMP.

Materials and Methods

Animal treatment and COC collection in experiment 1

Non-pregnant, non-lactating ewes (Western range ewes; primarily Rambouillet and Targhee) were randomly distributed into treatment groups as follows: non-treated (control, $n = 13$), FSH-treated for 2 days ($n = 9$) or FSH-treated for 3 days ($n = 8$). Beginning on the morning of day 13 of the estrous cycle (normal duration of the estrous cycle in our sheep flock is about 16.5 days), FSH-treated ewes received twice daily (morning and evening) intramuscular injections of FSH-P (FSH with 10% luteinizing hormone; Sioux Biochemical, Sioux Center, IA, USA). Doses were as follows: first day of FSH treatment, 5 units/injection; second day of FSH treatment, 4 units/injection; third day of FSH treatment, 3 units/injection (total dose: 2-day treated, 18 units; 3-day treated, 24 units; 1 unit is equivalent to 3.5 g of NIDDK-oFSH-20). On day 15 or day 16 of the estrous cycle, ewes were ovariectomized. For each follicle, the surface diameter was measured and follicles were classified as small (≤ 3 mm) or large (> 3 mm). Small follicles represent the early stage of follicular development, shortly after antrum formation, and large follicles represent the advanced, preovulatory stage of folliculogenesis. Cystic follicles were not present in any ovaries in this study. COCs were isolated by opening each visible follicle with a no. 15 scalpel blade and flushing it two to three times with a Pasteur pipette containing oocyte

collection medium (TCM-199 medium (Sigma), 2% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), heparin (100 U/ml; Sigma) and penicillin–streptomycin (10 000 units/ml penicillin, 10 000 µg/ml streptomycin; Gibco) into a petri dish. COCs were collected first from large follicles on a 60 mm petri dish, then ovaries were transferred to another petri dish for collection of COCs from small follicles. For day 15 of the estrous cycle, half the COCs were immediately embedded in agarose gel and then fixed in Carnoy's solution. The other half were incubated in maturation medium (TCM-199, 10% FBS, 5 µg/ml ovine FSH (oFSH-RP-1; NIAMDD-NIH, Bethesda, MD, USA), 5 µg/ml ovine LH (oLH-26; NIADDK-NIH), 1 µg/ml estradiol (0.5 µl/ml; Sigma), 2 mM glutamine (Sigma), 0.25 mM sodium pyruvate (Sigma), and penicillin–streptomycin (doses as above) Stenbak *et al.* 2001) for 21–24 h at 39°C, 5% CO₂ and 95% air, at 100% humidity under mineral oil stabilized for 24 h at conditions listed above, and then embedded in agarose gel and fixed in Carnoy's solution. For day 16 of the estrous cycle, COCs were embedded in agarose gel and fixed immediately after collection. For all FSH-treated ewes, and half of non-treated ewes and each category of follicle size, two to four oocytes (14–50% of total collected oocytes/ewe per follicle size) were used to evaluate maturation status before or after maturation. For non-treated (control) ewes, the stage of oocyte maturation was evaluated for half of the ewes because these animals have a low number of large follicles (one to four large follicles per ewe).

Animal treatment and COC collection in experiment 2

On days 13 and 14 of the estrous cycle, ewes ($n = 31$; breed as in experiment 1) received intramuscular injections of FSH-P twice daily as described above. Laparotomy was performed on day 15 of the estrous cycle and the ovaries were removed. For each follicle, the surface diameter was measured and follicles were classified as small (≤ 3 mm) or large (> 3 mm) COCs were collected from all small and large antral follicles as in experiment 1 and used for evaluation of GJIC of cumulus cells *in vitro*.

The protocols for procedures used in this study and for animal care were approved by the Institutional Animal Care and Use Committee at NDSU.

Embedding and sectioning procedures

COCs were embedded in a 1% (w/v) solution of low-melting-point agarose gel (Gibco) in PBS. A small piece of the gel containing the COC was cut out and fixed in Carnoy's solution for 30 min, washed in 70% ethanol, immersed in eosin solution (0.25% in 70% ethanol, w/v) for 20–30 min and then transferred to 70% ethanol. The gel containing the COC was embedded in paraffin. The paraffin blocks of COC were then sectioned at 4 µm.

Oocyte staining to determine maturation status

Cumulus cells were removed from oocytes after 5–10 min incubation at 37°C with hyaluronidase (0.1% in saline; Sigma) by using a micropipette with a diameter of 125 µm. Then, oocytes were fixed in methanol, stained with 0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA) in methanol for 15 min and then mounted on slides (Jablonka-Shariff & Olson 2000). The evaluation of nuclear status was done by epifluorescence microscopy. Oocytes in the germinal vesicle stage, containing diplotene chromatin, were considered to be immature. Oocytes demonstrating extrusion of the first polar body, which indicates metaphase II stage, were considered to be mature (Gaudet *et al.* 1997).

Immunohistochemical procedures

COC sections were stained for detection of Cx43 protein as described previously (Grazul-Bilska *et al.* 1998). The sections were treated with blocking buffer consisting of PBS containing 0.3% (v/v) Triton X-100 (Mallinckrodt, Paris, KY, USA) and 1% (v/v) normal goat serum (Vector Labs, Burlingame, CA, USA), followed by overnight incubation with rabbit polyclonal antibody against Cx43 (a gift from Dr W.J. Larsen, University of Cincinnati, OH, USA). In order to detect primary antibodies, biotinylated secondary antibody (goat antirabbit IgG; Zymed, San Francisco, CA, USA) and the ABC system (Vectastain; Vector Labs) were used. The sections were then counterstained with nuclear fast red (Sigma). For control sections the primary antibody was omitted. Control sections did not exhibit any staining (data not shown).

Image analysis

The area of positive staining for Cx43 (visible as black punctate staining on cell borders) and the total nuclear area (represented as pink stained areas) (Fig. 1), were determined for each COC using computerized image analysis. The total nuclear area was divided by the average area of each nucleus to obtain the total number of cumulus cells in each COC section. Cx43 data are expressed as the area of positive staining (µm²) per cumulus cell. Image analysis was performed using Image Pro-plus software (Media Cybernetics Inc., Silver Springs, MD, USA).

Culture of intact COCs and isolated cumulus cells

To get sufficient numbers of COCs and cumulus cells per treatment per follicle size, COCs from two to three ewes were pooled. For each culture, a portion of the COC pool was left intact, and another portion was treated with hyaluronidase (0.1%, w/v; Sigma) for 10–20 min at 37°C to obtain isolated cumulus cells. Intact COCs ($n = 3\text{--}4$ COCs/treatment group) and isolated cumulus cells (8×10^3 to 10×10^3 cells per treatment group) were cultured with or without FSH (100 ng/ml; NIADDK oFSH-RP-1), (100 ng/ml;

NIADDK oLH-26) or cAMP agonist (dbcAMP, 1 mM; Sigma) for 36–48 h at 39°C in Dulbecco's modified Eagle medium (DMEM; Gibco) with 20% FBS and 1% penicillin–streptomycin on 35 mm petri dishes coated with fibronectin (Sigma).

Evaluation of GJIC

GJIC among cumulus cells in culture was determined using a dye-coupling, fluorescence recovery after photobleaching (FRAP) technique and an interactive laser cytometer (ACAS 570; Meridian Instruments, Okemos, MI, USA; Redmer *et al.* 1991). Briefly, after incubation with the treatments, the medium was removed from the dish and fresh serum-free DMEM containing a fluorescent probe (calcein, 1 µM; Molecular Probes) was added. After 20–30 min of incubation, dishes were rinsed twice with serum-free DMEM to remove the excess dye. Dishes were then placed onto the laser cytometer, and three to four fields on each dish were identified for scanning. Immediately after measurement of initial fluorescence, the fluorescent probe was photobleached in several of the selected cells in each field ($n = 3\text{--}6$ cells/field). To determine rates of GJIC, the fluorescence intensity of all (photobleached and non-photobleached) selected cells was quantified 4 and 8 min after photobleaching using the FRAP procedure. GJIC was evaluated on the basis of the rate of recovery of fluorescence expressed as percentage recovery of fluorescence per minute (Redmer *et al.* 1991). Data are presented as the rate of FRAP (percentage of pre-bleach values per minute) calculated from values obtained during the first 4 min after photobleaching.

Statistics

Data for positive Cx43 staining per cell and the rates of GJIC of cumulus cells were analyzed separately using the general linear model (GLM) procedures of SAS (Statistical Analysis System Institute 1985). When an *F*-test was significant, differences between specific means were evaluated using Bonferroni's *t*-test (Kirk 1982).

Results

Experiment 1

Immunohistochemical staining in COCs

Positive punctate Cx43 staining (Fig. 1) was detected between adjoining cumulus cells, and between the oocyte and cumulus cells in all COCs irrespective of follicular size, maturation status and treatment group. In addition, some of the Cx43 positive staining was localized on the oocyte side of the zona pellucida (Fig. 1A, top insert).

The mean area of positive Cx43 staining per cumulus cell was similar in COCs from small (0.36 ± 0.02 µm²/cell) and large (0.33 ± 0.02 µm²/cell) follicles. However, the mean area of Cx43 staining in COCs was

Figure 1 Representative photomicrograph of Cx43 staining in immature (A) and mature (B) COCs. Upper insert in panel (A) demonstrates positive staining on the oocyte side of the zona pellucida in the twice-enlarged area marked with a rectangle in (A). Note dark, punctuate Cx43 positive staining (arrows) on the cellular borders. Bottom inserts in (A) and (B) demonstrate a positive Cx43 staining extracted from respective image by image analysis system. Control sections (upper insert in (B)) showed no positive staining. Magnification, $\times 600$.

greater ($P < 0.01$) on day 16 than on day 15 of the estrous cycle (0.71 ± 0.09 vs $0.16 \pm 0.03 \mu\text{m}^2/\text{cell}$; Fig. 2) in non-treated ewes. Compared with non-treated controls, a 3-day FSH treatment decreased ($P < 0.02$) Cx43 expression on day 16 ($0.71 \pm 0.09 \mu\text{m}^2/\text{cell}$ for control vs $0.24 \pm 0.05 \mu\text{m}^2/\text{cell}$ for 3-day FSH treatment; Fig. 2). In contrast, a 2-day FSH treatment increased Cx43 expression on day 15 ($0.16 \pm 0.03 \mu\text{m}^2/\text{cell}$ for control vs

$0.27 \pm 0.3 \mu\text{m}^2/\text{cell}$ for 2-day FSH treatment; Fig. 2). The Cx43 expression tended to be greater ($P < 0.1$) in non-mature COCs ($0.36 \pm 0.06 \mu\text{m}^2/\text{cell}$) than in mature COCs ($0.31 \pm 0.03 \mu\text{m}^2/\text{cell}$).

Evaluation of maturation status

On day 15, immediately after collection, all COCs ($n = 40$) had compacted layers of cumulus cells and oocytes were

Figure 2 Cx43 expression in COCs in non-treated and FSH-treated ewes on days 15 and 16 of estrous cycle, for each group 10–18 COCs were evaluated. Values ($\pm$ S.E.M.) with different superscripts differ ($P < 0.01$) within a day.

in germinal vesicle (GV) stage (Fig. 3A), but after overnight maturation, 95% of oocytes reached metaphase II stage (Fig. 3B). On day 16, all oocytes with compacted non-expanded cumulus cells were in GV stage ($n = 36$), but oocytes with expanded cumulus cells were in metaphase II stage ($n = 8$). However, the expanded COCs from day 16 were not included in this study because we obtained insufficient number of expanded COCs.

Experiment 2

GJIC

After incubation with the fluorescent dye, all cumulus cells and oocytes across all groups and treatments exhibited the presence of the dye. Following photobleaching, the cumulus cells showed a decline in fluorescence, which was followed by a recovery in all cells that were in contact with other cells (e.g. cell 1 in Fig. 4). Photobleached cumulus cells that were not in contact with other cells did not recover fluorescence (e.g. cell 3 in Fig. 4).

For cumulus cells in intact COCs and isolated cumulus cells, the basal (control) rate of GJIC measured as FRAP

was greater ($P < 0.01$) for large follicles than small follicles (3.7 ± 0.2 vs $2.2 \pm 0.1\%$ per min and 2.7 ± 0.1 vs $2.4 \pm 0.1\%$ per min respectively; Fig. 5).

For large follicles, the basal rate of GJIC was greater ($P < 0.01$) for cumulus cells in intact COCs ($3.7 \pm 0.2\%$ per min) than for isolated cumulus cells ($2.7 \pm 0.1\%$ per min; Fig. 5). Compared with control, LH and FSH decreased ($P < 0.01$) GJIC in intact COCs from large follicles (Fig. 5A). However, compared with control, FSH ($P < 0.04$) increased but LH had no effect on GJIC in intact COCs from small follicles (Fig. 5A). Compared with control, LH decreased ($P < 0.02$) but FSH did not affect GJIC of isolated cumulus cells from large follicles (Fig. 5B). However, compared with control, LH increased ($P < 0.01$) and FSH tended ($P < 0.1$) to increase GJIC of cumulus cells in isolated cumulus cells from small follicles (Fig. 5B).

Compared with control, dbcAMP increased ($P < 0.01$) GJIC of cumulus cells of small and large follicles irrespective of the presence or absence of oocyte (Fig. 5).

Discussion

Follicular somatic cells support oocyte development, and oocytes affect somatic cell function via metabolites transferred through gap junctions and/or other pathways (Eppig 1991, 2001, Granot & Dekel 2002, Sutton *et al.* 2003). It is well established that cell-to-cell communication in the ovarian follicles is mediated by channels predominantly comprising of Cx43 (Grazul-Bilska *et al.* 1997, Juneja *et al.* 1999, Granot & Dekel 2002). In the present study, Cx43 was localized between cumulus cells and between the oocyte and cumulus cells, and, in addition some of the staining was localized to the oocyte side of the zona pellucida. Similar to our findings, in most species Cx43 has been localized at the cellular borders of granulosa/cumulus cells and at the interface of the oocyte and the surrounding cumulus cells (Valdimarsson *et al.* 1993, Mayerhofer & Garfield 1995, Simon *et al.* 1997, Grazul-Bilska *et al.* 1998, Lenhart *et al.* 1998, Johnson *et al.* 1999, Nuttinck *et al.* 2000, Granot & Dekel 2002). Collectively, these findings demonstrate that Cx43 is present

Figure 3 Representative micrographs of oocytes in germinal vesicle stage (A), and in metaphase II stage (B). Magnification, $\times 400$.

Figure 4 Computer-generated fluorescent images of ovine COCs in culture. Note the presence of the oocyte (O). Fluorescent images are presented for: (A) cumulus cells in COCs immediately before photobleaching; (B) immediately after photobleaching; (C) 4 min after photobleaching. (D) FRAP for specific cells; cell numbers relate to white octagons on panels (A)–(C). Note that the fluorescence intensity of cell 2, which was not photobleached, did not change. Cell 1 was photobleached but was in contact with other cells and recovered much of its fluorescence. Cell 3 was photobleached but was not in contact with other cells and did not recover its fluorescence.

between the oocyte and the adjacent cumulus cells as well as in the oolemma.

The expression of Cx43 and the rate of GJIC in COCs seem to depend on the stage of follicular development (Mayerhofer & Garfield 1995, Grazul-Bilska *et al.* 1998, Johnson *et al.* 1999, 2002, Melton *et al.* 2001). Cx43 has been detected in primordial to preovulatory follicles and its expression has been shown to accompany follicular growth in rats (Mayerhofer & Garfield 1995) and pigs (Lenhart *et al.* 1998). For sheep and cows, an increased Cx43 expression has been reported in large antral follicles as compared with small antral follicles (Grazul-Bilska *et al.* 1998, Johnson *et al.* 1999, 2002, Nuttinck *et al.* 2000). Although we observed a more intense Cx43 staining in COCs of large follicles compared with small follicles in the present experiment, the area of positive staining remained similar for large and small follicles. However, Cx43 expression in COCs was greater on day 16 than on day 15 of the estrous cycle. Moreover, in the current study, GJIC was greater in cumulus cells derived from large follicles as compared with small follicles. The increase in gap junction function with follicle size might be related to the increasing demands of the growing follicle for enhanced exchange of metabolites and regulatory signals through contact-dependent pathways.

The maturation status of the oocyte is subject to regulation by the somatic compartment of the ovarian follicle

via transfer of regulatory molecules (Eppig 1991, Downs 1995). In our study, mature expanded COCs showed a tendency for reduced Cx43 expression as compared with immature non-expanded COCs. A similar decrease of Cx43 expression in cumulus cells was observed after oocyte maturation in mares (Marchal *et al.* 2003). Previous studies in rodents have established that the onset of meiotic maturation is preceded by the breakdown of gap junctions in the follicles (Larsen *et al.* 1986, Downs 1995), which restricts the flow of signals from follicular cells to the oocyte. However, we observed the presence of Cx43 in COCs with expanded cumulus cells, implying that some level of communication remains between the oocyte and cumulus cells after maturation. The presence of gap junctional coupling in COCs post maturation, as evidenced by the Cx43 expression in mature COCs, might indicate that the communication between the oocyte and the cumulus cells and/or among the cumulus cells via gap junctions is necessary for providing the metabolic support, and possibly for oocyte pick-up by the infundibulum, transport of the oocyte through the oviduct and fertilization (Sutovsky *et al.* 1993, Eppig *et al.* 1997, Lam *et al.* 2000). However, this subject requires further studies.

In the current study, the presence of the oocyte in intact COCs enhanced the GJIC of cumulus cells as compared with isolated cumulus cells. In several species, a specific role of the oocyte in the regulation of granulosa/cumulus

Figure 5 Effects of FSH, LH and cAMP on GJIC of cumulus cells in intact COC (A) and isolated cumulus cell (B) cultures derived from large and small follicles. Numbers at the bottom of each bar indicate numbers of evaluated cells. * $P < 0.05$; values ($\pm$ S.E.M.) differ from control within follicle size.

cell function *in vitro* has been established (Vanderhayden *et al.* 1992, Eppig 2001, Sutton *et al.* 2003). It has been demonstrated that the oocyte affects the proliferation of granulosa cells, the maintenance of structural organization of the ovarian follicle, and aids in cumulus expansion and synthesis of hyaluronic acid in response to FSH or cAMP (Vanderhayden *et al.* 1992, Singh *et al.* 1993). Our findings indicate that in addition to the above listed functions, the oocyte influences gap junction function in COCs.

Gonadotropins have been documented to affect ovarian function including gap junction expression and function throughout the estrous cycle (Wiesen & Midgley 1993, Grazul-Bilska *et al.* 1997, Granot & Dekel 2002, Webb *et al.* 2002). The expression of Cx43 in the ovarian follicle has been associated with the profile of serum concentrations of FSH and LH (Wiesen & Midgley 1993, Granot & Dekel 1997). Numerous studies have demonstrated the specific effects of FSH and LH on gap junction presence, Cx43 expression and GJIC in the ovarian follicle (Burghardt & Matheson 1982, Mayerhofer & Garfield 1995, Grazul-Bilska *et al.* 1996, 1997, Granot & Dekel 1997, Sommerburg *et al.* 2000). Both FSH and LH affect the function of granulosa/cumulus cells directly by binding to specific receptors (Greenwald & Roy 1994).

We observed a significant increase in Cx43 expression on day 16 of the estrous cycle in ewes as compared with

day 15, which suggests that higher endogenous FSH concentrations (Goodman 1994) may have increased Cx43 expression. In addition, the administration of FSH for 2 days increased the expression of Cx43 on day 15 of the estrous cycle but decreased it on day 16 following a 3-day regimen of FSH. This demonstrates that the duration of FSH treatment affects Cx43 expression in COCs.

Several studies have demonstrated both positive and negative effects of FSH on oocyte morphology and quality in sheep (Moor *et al.* 1985, O'Callaghan *et al.* 2000). Moor *et al.* (1985) reported that exogenous FSH administration at high levels could disrupt the biochemical functions of oocytes causing them to undergo premature activation, resulting in the oocytes being already aged at the time of ovulation or collection. Previous studies in our laboratory have indicated that *in vivo* FSH administration for 3 days results in a decline in the cleavage rate as compared with a 2-day treatment in ewes (Stenbak *et al.* 2001). Since we observed a decrease in Cx43 expression after a 3-day treatment with FSH, we can suggest that disruption of gap junction function by prolonged FSH treatment is affecting oocyte quality; however, this subject requires further study.

In the current study, FSH and LH affected GJIC of cumulus cells in a stage-specific manner. For small follicles, we observed the stimulatory effects of gonadotropins on GJIC of cumulus cells; however, for large follicles, we observed rather inhibitory effects of gonadotropins on GJIC of cumulus cells.

In small follicles, an increased GJIC following incubation with FSH may be due to the high expression of FSH receptors that may permit coupling of FSH (Yuan *et al.* 1996). The stimulatory effects of FSH on GJIC have been demonstrated for bovine granulosa cells (Johnson *et al.* 2002) and for a rat granulosa cell line (Sommerburg *et al.* 2000). Stimulation by FSH is followed by an elevation of cAMP in granulosa/cumulus cells (Salustri *et al.* 1985, Webb *et al.* 2002). It has been demonstrated that the exposure of granulosa cells to FSH increases the intercellular communication by activation of the cAMP-dependent protein kinase A (PKA) pathway leading to the clustering of junctional channels into functional gap junctions (Godwin *et al.* 1993, Grazul-Bilska *et al.* 1997). The absence of the effect of FSH on GJIC of cumulus cells from large follicles might be associated with a decreased number of FSH receptors (Liu *et al.* 1998, Cardenas & Pope 2002).

In the present experiment, LH inhibited GJIC for both isolated cumulus cells and in intact COC from large follicles, and increased GJIC of isolated cumulus cells from small follicles. Exposure of granulosa/cumulus cells to LH leads to an elevation in cAMP and causes stimulation of the PKA pathway (Gudermann *et al.* 1992, Morris & Richards 1993). In addition, the binding of LH to its receptors generates diacylglycerol, which activates the PKC pathway (Gudermann *et al.* 1992, Morris & Richards 1993). The hyperphosphorylation of gap junctional proteins when exposed to PKC may lead to the closure of junctional

channels (Granot & Dekel 2002). Since there are very few LH receptors in the small follicles, it is possible that both kinase cascades cannot be stimulated and only PKA is activated, which leads to an increase in GJIC. However, in large follicles, LH may also activate the PKC pathway, which probably leads to gap junctional uncoupling. Granot and Dekel (2002) demonstrated that the uncoupling effects of LH are mediated via a large increase in cAMP which stimulates protein kinases A and C cascades.

The preovulatory surge of serum LH is associated with a decrease in cellular communication in the COC, the decrease in Cx43 mRNA and protein expression resulting in a substantial reduction in membrane areas of gap junction (Wiesen & Midgley 1993, Granot & Dekel 1997). In addition, exposure of the follicle to LH or human chorionic gonadotropin (hCG) causes the interruption of ionic coupling between granulosa/cumulus cells and the oocyte (Larsen *et al.* 1981, 1986). In agreement with other researchers, we saw a decrease in GJIC of cumulus cells from large follicles following incubation with LH (Larsen *et al.* 1981, 1986). Several studies have indicated that in oocytes nearing ovulation, there is a decrease in junctional coupling (Larsen *et al.* 1986, Chen *et al.* 1990). This effect may be due to an unidentified signal from the oocyte to the granulosa/cumulus cells that overrides the usual coupling-enhancing effect of FSH. These data demonstrate that the effects of FSH and LH on GJIC of cumulus cells depend on the stage of follicular development.

In the current study, cAMP increased GJIC of cumulus cells across all groups. The ability of cAMP to upregulate gap junction coupling has been demonstrated in numerous cell types including ovarian cells (Grazul-Bilska 1996, 1997, Luciano *et al.* 2004, Thomas *et al.* 2004). Prolonged coupling of cells in the presence of cAMP has been shown to increase the rate of synthesis of connexin mRNA or to increase stability of the transcript, thereby generating a higher rate of synthesis of gap junction protein available for assembly into new channels (Mehta *et al.* 1992). cAMP is also postulated to induce gap junctional permeability by reapportioning Cx43 from non-junctional sites to junctional plaques at cell–cell interfaces and increase gap junction formation and membrane permeability (Azarnia *et al.* 1981, Atkinson *et al.* 1995). cAMP activates protein kinase pathways which phosphorylate several proteins including connexin (Granot & Dekel 2002). Basal phosphorylation of connexins is mandatory to maintain the open state of junctional channels (Godwin *et al.* 1993) and hence the GJIC-enhancing effect of cAMP could be mediated via phosphorylation of channel proteins. These results indicate that cAMP stimulates GJIC in ovarian follicles, possibly via several mechanisms (listed above), irrespective of follicular size or presence of oocyte.

In summary, Cx43 is localized on the cellular borders of cumulus cells as well as between the oocyte and surrounding cumulus cells. The day of the estrous cycle,

stage of maturation and duration of FSH treatment affect the expression of Cx43 in ovine COCs. The oocyte appears to exert a positive effect on the GJIC of cumulus cells in this study. However, an additional study should be undertaken to define further and assess adequately a specific role of oocyte in regulation of GJIC of cumulus cells. The junctional coupling of cumulus cells is associated with follicular growth and increases as follicular size increases. In addition, the GJIC of cumulus cells is influenced by gonadotropins, and the effect of gonadotropins is dependent on the stage of development of the follicle. cAMP upregulates the GJIC of cumulus cells irrespective of the stage of follicular growth or the presence of oocyte.

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