Role of gap junctions in regulation of progesterone secretion by ovine luteal cells *in vitro*

Ewa Borowczyk¹, Mary Lynn Johnson^{1,2,3}, Jerzy J Bilski^{1,2}, Magda A Bilaska¹, Dale A Redmer^{1,2,3}, Lawrence P Reynolds^{1,2,3} and Anna T Grazul-Bilska^{1,2,3},

¹Department of Animal and Range Sciences, ²Cell Biology Center and ³Center for Nutrition and Pregnancy, North Dakota State University, Fargo, North Dakota 58105, USA

Correspondence should be addressed to A T Grazul-Bilska; Email: anna.grazul-bilska@ndsu.edu

Abstract

To evaluate the role of gap junctions in the regulation of progesterone secretion, two experiments were conducted. In Experiment 1, luteal cells obtained on days 5, 10, and 15 were cultured overnight at densities of 50×10^3 , 100×10^3 , 300×10^3 , and 600×10^3 cells/dish in medium containing: (1) no treatment (control), (2) LH, or (3) dbcAMP. In Experiment 2, luteal cells from days 5 and 10 of the estrous cycle were transfected with siRNA, which targeted the connexin (Cx) 43 gene. In Experiment 1, progesterone secretion, Cx43 mRNA expression, and the rates of gap junctional intercellular communication (GJIC), were affected by the day of the estrous cycle, cell density, and treatments (LH or dbcAMP). The changes in progesterone secretion were positively correlated with the changes in Cx43 mRNA expression and the rates of GJIC. Cx43 was detected on the luteal cell borders in every culture, and luteal cells expressed 3β -hydroxysteroid dehydrogenase. In Experiment 2, two Cx43 gene-targeted sequences decreased Cx43 mRNA expression and progesterone production by luteal cells. The changes in Cx43 mRNA expression were positively correlated with changes in progesterone concentration in media. Thus, our data demonstrate a relationship between gap junctions and progesterone secretion that was supported by (1) the positive correlations between progesterone secretion and Cx43 mRNA expression and GJIC of luteal cells and (2) the inhibition of Cx43 mRNA expression by siRNA that resulted in decreased production of progesterone by luteal cells. This suggests that gap junctions may be involved in the regulation of steroidogenesis in the ovine corpus luteum.

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Introduction

The mammalian corpus luteum (CL) is a dynamic, transient endocrine gland and a major source of progesterone, which is critical for reproductive cyclicity and maintenance of pregnancy (Milvae *et al.* 1996, Pate 1996, Reynolds & Redmer 1999, Niswender *et al.* 2000). In fact, inadequate luteal function has been shown to be one of the major causes of reproductive failure (Juengel & Niswender 1999, Reynolds *et al.* 2002).

The corpus luteum is a complex, heterogeneous tissue composed of several cell types including steroidogenic (parenchymal) and nonsteroidogenic (nonparenchymal) cells (Milvae *et al.* 1996, Pate 1996). Cellular interactions mediated through contact-independent (humoral) and contact-dependent (gap junctional) pathways within the CL are believed to be essential for the maintenance of normal luteal tissue function (Redmer *et al.* 1991, Del Vecchio *et al.* 1994, 1995a,

1995b, Redmer & Reynolds 1996, Grazul-Bilska *et al.* 1997a, 1997b).

Gap junctional intracellular communication (GJIC) is involved in numerous physiological processes, including endocrine and exocrine secretions, providing a functional integration and coordination of activity within various glands (Meda *et al.* 1993, Munari-Silem & Rousset 1996, Serre-Beinier *et al.* 2002). By facilitating the transport of nutrients, ions, and regulatory molecules less than 1 kDa between adjacent cells, gap junctions have been demonstrated to play a fundamental role in regulation and coordination of cellular and/or tissue functions, including those in luteal tissues (Grazul-Bilska *et al.* 1997a, 1997b, Sohl & Willecke 2004). In fact, several connexins are expressed in endocrine glands, with Cx43 identified as the most widespread 'endocrine' connexin (Meda *et al.* 1993, Morand *et al.* 1996, Munari-Silem & Rousset 1996, Grazul-Bilska *et al.* 1997a, Serre-Beinier *et al.* 2002).

Functional and structural gap junctions present in luteal cells are regulated by systemic and local regulators of luteal tissue function, such as hormones, growth factors, cytokines and second messengers (Grazul-Bilska *et al.* 1997a, 1997b, 2001). For several species, it has been demonstrated that luteinizing hormone (LH) and dbcAMP increase the rate of GJIC between luteal cells and progesterone secretion (Redmer *et al.* 1991, Grazul-Bilska *et al.* 1997a, 1997b). Moreover, Cx43 seems to be the major connexin within luteal tissues that is expressed in the CL during the estrous cycle. Cx43 is localized to the luteal cell borders, therefore, Cx43 likely forms gap junctional channels allowing for GJIC (Grazul-Bilska *et al.* 1997a, 1997b).

Previously, we have demonstrated an association between progesterone secretion and GJIC (Grazul-Bilska *et al.* 1998a, 2001). To further investigate this relationship between progesterone secretion and gap junction function, we hypothesized that cell–cell contact would affect basal and/or induced progesterone secretion and that inhibition of Cx43 using siRNA would decrease progesterone secretion by luteal cells during the estrous cycle. Therefore, the objectives of this *in vitro* experiment were to: (1) determine the effects of cell–cell contact on basal and LH- or dbcAMP-induced progesterone secretion, Cx43 expression, and GJIC of luteal cells and (2) evaluate the effects of Cx43 siRNA on progesterone secretion and Cx43 mRNA expression in luteal cells collected throughout the estrous cycle in sheep. We used a cell density culture model, which creates a possibility of formation of more (confluent cultures) or less (sub-confluent cultures) gap junctional channels among cells.

Materials and Methods

Animal treatment and tissue collection

The protocols and animal care for this study were approved by the Institutional Animal Care and Use Committee at North Dakota State University. Crossbred ewes that exhibited at least one estrous cycle of normal duration (15–17 days) were treated twice daily (morning and evening) with i.m. injections of follicle stimulating hormone on days 13, 14, and 15 of the estrous cycle according to the protocols used previously (Grazul-Bilska *et al.* 2001). Day 0 of the estrous cycle (standing estrus) was determined using vasectomized rams.

Luteal tissue dispersion and cell separation

In Experiment 1, ovaries were collected from superovulated ewes, on days 5, 10, and 15 ($n=5$ ewes/day) of the estrous cycle, and in Experiment 2, ovaries were collected on day 5 ($n=4$) and day 10 ($n=4$) of the estrous cycle. Superovulation was necessary to collect multiple CL from each ewe for all analyses. McClellan

et al. (1975) and Hild-Petito *et al.* (1987) demonstrated that CL from superovulated and non-superovulated ewes are comparable in hormonal production and cellular composition. Corpora lutea ($n=4$ –34 from each ewe; average 16.5 ± 1.8 /ewe) were dissected from the ovaries, and luteal tissue was separated from the connective tissue and cut into small pieces. Luteal tissue was dispersed using Hanks' Balanced Salt Solution (HBSS; Sigma) containing collagenase type 4 (0.2% wt/vol; Worthington, Biomedical Corporation, Lakewood, NJ, USA), BSA (2% (wt/vol); Sigma), and antibiotics (100 U penicillin and 100 µg streptomycin/ml; Gibco, Grand Island, NY, USA) as described previously (Grazul-Bilska *et al.* 2001). Dispersed luteal cells were resuspended in Dulbecco's modified Eagle plating medium (DMEM) containing 1% fetal bovine serum (FBS; v/v; Gibco), 1% calf serum (CS; v/v; Gibco), and antibiotics (100 U penicillin and 100 µg streptomycin/ml; Gibco).

Culture of luteal cells

Experiment 1

Cells were counted by using a hemocytometer, and plated at concentrations of 50×10^3 , 100×10^3 , 300×10^3 , and 600×10^3 steroidogenic cells on 35 mm petri dishes (10–12 dishes for each density and treatment for each ewe) in DMEM with 1% FBS, 1% CS, and penicillin/streptomycin for overnight preincubation at 37 °C in a humidified atmosphere (5% CO₂:95% air) to facilitate cell attachment. Then, plating medium was changed to serum-free medium (Redmer *et al.* 1991), without (control), or with LH (100 ng/ml; United States Department of Agriculture bovine LHb5; Animal Hormone Program and the National Hormone and Pituitary Program, Beltsville, MD, USA) or dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP, 1 mM; analog of cyclic AMP; Sigma). The dose of LH or dbcAMP was chosen on the basis of previous experiments (Grazul-Bilska *et al.* 1996a, 2001). After 24 h incubation with treatments, GJIC of luteal cells was evaluated, medium was collected for progesterone determination, and cells were collected, resuspended in Trizol (Molecular Research Center, Cincinnati, OH, USA) and then stored at –70 °C until RNA was extracted. In addition, two sets of dishes from each culture were fixed for immunocytochemical detection of Cx43 protein and histochemical detection of 3β-hydroxysteroid dehydrogenase (3β-HSD). Pictures were taken from 3β-HSD stained cultures in order to determine a proportion of cells in contact with other cell(s).

Experiment 2

Dispersed luteal cells were plated in eight-chamber slides (50 000 cells/chamber) as described for Experiment 1. After overnight preincubation of luteal cells as described above, but before transfection, cells were washed three

times with Opti-minimum essential medium (MEM) (Gibco) with 2% serum without antibiotics.

Analysis of contact-dependent GJJC

Experiment 1

Evaluation of GJJC was conducted using a dye-coupling fluorescence recovery after photobleaching (FRAP) technique, and interactive laser cytometry (Redmer *et al.* 1991, Grazul-Bilska *et al.* 1996a, 2001). Briefly, after incubation with treatments, medium was replaced with fresh serum-free medium containing the fluorescent probe, calcein-AM (10 μ M; Molecular Probes; Eugene, OR, USA) and incubated for 10 min at 24 °C. Then, dishes were washed with serum-free medium in order to remove the excess of calcein and were placed onto the interactive laser cytometer. Three fields (180 $\times$ 180 μ m/field) on each dish were identified for scanning, 6–12 cells were selected within each field and analyzed for initial fluorescence intensities. The fluorescent probe was photobleached in four to eight selected cells in each field. After photobleaching, the fluorescence intensity of all selected cells was quantified after 4 and 8 min in order to determine the rate of FRAP and only the linear portion of the fluorescence recovery curve was chosen for the evaluations (the first 4 min after photobleaching; Redmer *et al.* 1991, Grazul-Bilska *et al.* 2001).

Immunohistochemistry

Experiment 1

The presence of Cx43 in luteal cell cultures was visualized using the immunohistochemical method described previously (Grazul-Bilska *et al.* 2001). Briefly, luteal 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 consisting of PBS (0.01 M phosphate and 0.14 M NaCl (pH 7.3)) containing 0.3% (v/v) Triton X-100 and 1% (v/v) normal goat serum, and then incubated overnight at 4 °C with a rabbit polyclonal antibody against Cx43 (Zymed Laboratories Inc., San Francisco, CA, USA). This antibody has been demonstrated to be specific for ovine Cx43 (Grazul-Bilska *et al.* 1998b). Detection of the primary antibody was accomplished using fluorescein isothiocyanate-conjugated secondary antibody (i.e., goat anti-rabbit IgG; Zymed Laboratories Inc.). Controls were incubated with normal rabbit serum instead of primary antibody.

Identification of steroidogenic cells

Experiment 1

To detect 3 β -HSD (marker of steroidogenic cells), cultured cells were fixed in 10% formalin for 10 min

followed by rinsing with distilled water and incubated with staining solution containing BSA (2 g/l), etiocholan-3 β -ol-17-one (58 mg/l; Sigma), nitroblue tetrazolium (204 mg/l; Sigma), and β -nicotinamide adenine dinucleotide (996 mg/l; Sigma) in PBS (0.01 phosphate, 0.14 NaCl, pH 7.3) overnight at 4 °C (Grazul-Bilska *et al.* 1996a). Staining solution without steroid was used for controls. Then, after rinsing with PBS, the stained area was covered with a coverslip and mounted with aqueous mounting medium (Gel-mount; Biomed, Foster City, CA, USA). Steroidogenic luteal cells containing 3 β -HSD exhibited dark staining.

Image analysis

Experiment 1

In order to determine the proportion of cells touching other cell(s) in each cell culture, ten randomly chosen fields from three dishes of each culture density, stained for the presence of 3 β -HSD, were photographed. The total number of cells and the number of cells touching other cells per field was determined using an image-analysis system (Image ProPlus, Media Cybernetics, Silver Spring, MD, USA). The data were reported as the percentage (mean $\pm$ S.E.M.) of cells touching other cell(s) out of the total number of cells per field.

Design of siRNA for Cx43

Experiment 2

Four targeted sequences (TS2, TS8, TS12, and TS24; Table 1), specific to Cx43 gene, were designed as potential siRNA target sites following the instructions from Ambion (Austin, TX, USA). DNA oligos representing the antisense siRNA strands (complement of the target) and the sense strands of siRNA (same sequence as the target mRNA) sequences were purchased from Dharmacon Research (Madison Inc, WI, USA; Table 1). For siRNA synthesis, sense and antisense strands of these siRNA templates were *in vitro* transcribed for 2 h, using T7 RNA polymerase, in separate reactions. Then, reactions for each targeted sequence were combined, mixed, and

Table 1 Sequence of siRNA for targeted sequences (TS) specific to Cx43.

TS ^a	siRNA	Nucleotide sequence
2	Antisense	5'-UCCUGCUACUGGGGACAGCtt-3'
	Sense	5'-GCUGUCCCCAGUAGCAGGAtt-3'
8	Antisense	5'-UCUCUCAUGUGCGCUUCUGtt-3'
	Sense	5'-CAGAAGCGCACAUAGAGAtt-3'
12	Antisense	5'-CAAGAAAGAGGAGGAACUCtt-3'
	Sense	5'-GAGUCCUCCUUCUUCUUGtt-3'
24	Antisense	5'-GAGCACGGCAAGGUGAAGAtt-3'
	Sense	5'-UCUUCACCUUGCCGUGCUCtt-3'

The antisense siRNA strand is the reverse complement of the target sequence, Cx43. The sense siRNA strand is the same sequence as the target mRNA sequence without the 5' AA sequence.

^aTS, targeted sequence.

incubated to facilitate hybridization. After overnight incubation, double-stranded (ds) siRNA for each targeted sequence were purified and used following the Mirus (Madison, WI, USA) transfection protocol.

Visualization of transfection

Experiment 2

To determine the transfection efficiency of luteal cells, fluorescently (Cy3) labeled TS8 siRNA purchased from Dharmacon Research Inc. was used (Fig. 1).

Transfection with siRNA

Experiment 2

Luteal cells were cultured in 250 µl Opti-MEM/DMEM (1:4) medium/well with 2% serum without antibiotics containing: no supplements (control), transfection

Figure 1 Representative micrographs of luteal cells transfected with 20 nM TS8 siRNA for Cx43 labeled with Cy3. Micrograph on the top is a light microscopic image of cultured luteal cells (differential interface contrast; Hoffman modulation) and on the bottom is a fluorescence microscopy image of the same area. Bright red fluorescence indicates transfected cells. Magnification 200×.

reagent (TransIT-TKO; Mirus) without siRNA, transfection reagent (TransIT-TKO; Mirus) with TS2, TS8, TS12 or TS24 (20 nM). After 24 h incubation with treatments, the morphology and viability of cells were evaluated, and then medium was collected for evaluation of progesterone concentration using RIA. The cells were collected and frozen for extraction of total cellular (tc) RNA for real-time PCR analysis.

RNA isolation and RT

Experiments 1 and 2

RNA was extracted from cultured cells using Trizol (Molecular Research Center) according to the manufacturer's recommendations. Polyacrylamide carrier solution was added to facilitate greater RNA yields. The Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used to determine the quantity and quality of total cellular RNA samples. All RNA samples were reverse transcribed in triplicate 20 µl reactions using Taqman RT Reagents (Applied Biosystems, Foster City, CA, USA). All cDNAs from the RT reaction were stored at -20°C prior to PCR analysis (Borowczyk *et al.* 2006a).

Real-time quantitative RT-PCR

Experiments 1 and 2

Cx43 mRNA expression was determined by quantitative real-time RT-PCR analysis using the ABI PRISM 7000 Sequence Detection System and software. Primers and probes were designed using the Primer Express software version 2.0 and cDNA from ovine heart was used as a standard curve (Borowczyk *et al.* 2006a). For an RNA control, 18S rRNA was analyzed using the same PCR protocols and the human 18S PDAR kit from Applied Biosystem. Then, Cx43 mRNA values were normalized to 18S rRNA by dividing them by their corresponding 18S rRNA values (Borowczyk *et al.* 2006a).

Progesterone RIA

Experiments 1 and 2

Concentrations of progesterone in culture media were measured according to the protocol used previously (Grazul-Bilska *et al.* 1996a, 2001). The sensitivity of the assay was 12.5 pg/tube and the intra- and interassay coefficients of variation were 4.5 and 8.9%, and 4.8 and 8.5%, for experiments 1 and 2 respectively.

Statistical analysis

Data for progesterone secretion by luteal cells, Cx43 mRNA expression and GIJC of luteal cells, and the

proportion of cells in contact with other cell(s) were analyzed using general lineal model ANOVA (SAS 2005). When an *F*-test was significant ($P < 0.05$), differences between means were evaluated with least square means procedure (Kirk 1982). Correlations between the progesterone concentration in medium, Cx43 expression and GJIC were evaluated using PROC CORR of SAS.

Results

In Experiment 1, the proportion of cells touching other cell(s) within each cell density culture was similar throughout the estrous cycle, therefore the data were combined (see below) for days 5, 10, and 15 of the estrous cycle. The greatest ($P < 0.01$) proportion of cells touching one or more cells was detected in cultures with 600×10^3 cells/dish, and gradually decreased ($P < 0.01$) in cultures with 300×10^3 , 100×10^3 , and 50×10^3 cells/dish (98.5 ± 1 , 83.24 ± 1.2 , 73.3 ± 1.4 , $51 \pm 1.7\%$ respectively) across the days of the estrous cycle or treatment.

Across all cell densities, basal progesterone secretion by luteal cells was greater ($P < 0.001$) on day 10 than on days 5 or 15 of the estrous cycle (414 ± 100 vs 198 ± 52 and 164 ± 43 ng/ml respectively). As expected, basal progesterone concentration in media was the lowest ($P < 0.001$) in cultures with densities of 50 or 100×10^3 cells/dish (54 ± 10 or 104 ± 21 ng/ml), greater ($P < 0.001$) in cultures with 300×10^3 cells/dish (282 ± 54 ng/ml) and the greatest ($P < 0.001$) in cultures with 600×10^3 cells/dish (594 ± 117 ng/ml) across the estrous cycle. When basal progesterone secretion was expressed per cell (progesterone concentration in 1 ml medium divided by cell number), progesterone levels were similar in cultures having a different cell density/dish when compared within the same day of the estrous cycle (Table 2). However, basal progesterone secretion expressed per cell was affected ($P < 0.05$) by the day of the estrous cycle (Table 2). Luteal cells from day 10 of the estrous cycle secreted more ($P < 0.05$) progesterone than cells from days 5 and 15 of the estrous cycle at cell densities 50×10^3 and 600×10^3 cells/dish (Table 2). However, for cell densities 100×10^3 and 300×10^3 cells/dish, progesterone secretion by luteal cells from

day 10 was similar to that on day 5 but greater ($P < 0.05$) than that on day 15 of the estrous cycle (Table 2).

Overall, LH and dbcAMP increased ($P < 0.001$) progesterone secretion by luteal cells in cultures with a different cell density from days 5, 10, and 15 of the estrous cycle. Since we did not observe the effects of cell density on LH- or dbcAMP-induced progesterone secretion expressed per cell, data for all cell densities are combined within a day of the estrous cycle (Fig. 2). LH increased ($P < 0.01$) progesterone secretion on day 5 and tended to increase progesterone secretion ($P < 0.1$) on days 10 and 15 of the estrous cycle. However, dbcAMP increased ($P < 0.01$) the progesterone secretion by luteal cells across all days of the estrous cycle (Fig. 2).

Across all treatments and cell densities, basal Cx43 mRNA expression by luteal cells was greater ($P < 0.001$) on day 10 than on days 5 and 15 of the estrous cycle (0.53 ± 0.07 vs 0.29 ± 0.04 or 0.33 ± 0.03 relative expression respectively). Basal Cx43 mRNA expression was affected ($P < 0.001$) by days of the estrous cycle and by cell density (Table 3). On days 5 and 10 of the estrous cycle, Cx43 mRNA expression by luteal cells was greater ($P < 0.05$), but on day 15 tended ($P < 0.1$) to be greater, in higher cell density cultures (300×10^3 or 600×10^3 cells/dish) than in lower cell density cultures (50×10^3 or 100×10^3 cells/dish; Table 3).

Overall, LH and dbcAMP increased ($P < 0.001$) Cx43 mRNA expression in luteal cells from cultures with a different cell density from different days of the estrous cycle. Since the effects of LH or dbcAMP on Cx43 mRNA expression were similar at all cell density cultures, data are combined for all cell densities within a day of the estrous cycle (Fig. 3). LH increased ($P < 0.05$) Cx43 mRNA expression on days 5 and 10, and dbcAMP increased ($P < 0.03$) and tended ($P < 0.1$) to increase Cx43 mRNA expression on days 5 and 10 respectively (Fig. 3). Cx43 mRNA expression by luteal cells from day 15 of the estrous cycle was not affected by LH or dbcAMP treatment (Fig. 3).

Across all treatments and days of the estrous cycle, the rate of GJIC was the greatest ($P < 0.001$) in cultures with 600×10^3 cells/dish ($1.7 \pm 0.03\%/min$), lower in cultures with 300×10^3 cells/dish ($1.5 \pm 0.03\%/min$), and 100×10^3 cells/dish ($1.0 \pm 0.02\%/min$), and least in cultures with 50×10^3 cells/dish ($0.9 \pm 0.02\%/min$). LH increased ($P < 0.05$) the rate of GJIC of luteal cells in cultures with

Table 2 Basal progesterone concentration in medium (expressed as pg/cell based on progesterone concentration in 1 ml medium) from different cell density cultures (50 – 600×10^3 cells/dish) on days 5, 10, and 15 of the estrous cycle.

Day of the estrous cycle	Cell density			
	50×10^3	100×10^3	300×10^3	600×10^3
5	$0.7 \pm 0.2^*$	$1.0 \pm 0.3^{*†}$	$0.8 \pm 0.2^{*†}$	$0.7 \pm 0.3^*$
10	$2.0 \pm 0.3^†$	$1.7 \pm 0.4^*$	$1.6 \pm 0.4^*$	$1.5 \pm 0.4^†$
15	$0.5 \pm 0.1^*$	$0.4 \pm 0.1^†$	$0.5 \pm 0.1^†$	$0.7 \pm 0.1^*$

Values are expressed as mean $\pm$ S.E.M. Individual basal values were obtained from each cell culture density without LH or dbcAMP-treatments.

* $†P < 0.05$, within a column means with different superscripts differ.

Figure 2 Effects of LH and dbcAMP on progesterone secretion by luteal cells from days 5, 10, and 15 of the estrous cycle. Data are presented as a percentage of the control (no treatment, 100%). Data are combined for all cell density cultures within a day of the estrous cycle. For basal progesterone production (pg/cell) in control (no treatment) cultures, see Table 2. Mean $\pm$ S.E.M. compared with control (no treatment; 100%) differ; * $P < 0.01$ and † $P < 0.1$.

densities of 300×10^3 and 600×10^3 , but not in cultures with 50×10^3 or 100×10^3 cells/dish on days 5 and 10 of the estrous cycle (Fig. 4). DbcAMP increased ($P < 0.05$) the rate of GJIC of luteal cells in cultures with densities of 100×10^3 , 300×10^3 , and 600×10^3 , but not with 50×10^3 cells/dish on day 5, and also in cultures with 300×10^3 and 600×10^3 , but not for cultures with 50×10^3 or 100×10^3 cells/dish on day 10 of the estrous cycle (Fig. 4). However, on day 15 of the estrous cycle, LH or dbcAMP did not affect the rate of the GJIC in any cell-density culture (Fig. 4). Across all cultures, there were no treatment or day of the estrous cycle effects on the rate of GJIC for cells, which were not photobleached (positive control, $n = 821$) or for cells, which were photobleached but not in contact with other cells (negative control, $n = 350$).

Overall, the progesterone secretion was positively correlated with Cx43 mRNA expression, and Cx43 mRNA expression was positively correlated with the rates of GJIC (Table 4). Moreover, changes in progesterone secretion caused by the day of the estrous cycle and the treatments were positively correlated with changes in Cx43 mRNA expression and GJIC of luteal cells throughout the estrous cycle ($R = 0.209$ and 0.257 , $P < 0.006$ respectively).

Cx43 protein was found on luteal cell borders (Fig. 5A and B), and most luteal cells in every culture expressed 3 β -HSD (Fig. 6A–D).

In Experiment 2, TS8 and 24, but not TS2 or 12, affected ($P < 0.05$) progesterone secretion and Cx43 mRNA expression by luteal cells from days 5 or 10 of the estrous cycle (Fig. 7). Since similar results were obtained for both the days, the data for days 5 and 10 were combined. Progesterone secretion and Cx43 mRNA expression were decreased ($P < 0.02$) by approximately 30 and 40% respectively, in the presence of siRNA for Cx43 in luteal cell cultures. These changes in Cx43 mRNA expression were positively correlated ($P < 0.01$) with changes in progesterone concentration in media ($R = 0.543$).

Discussion

The present study clearly shows an association between the major function of luteal cells, which is the secretion of progesterone, and gap junction function, since a positive correlation was demonstrated between progesterone secretion, Cx43 mRNA expression, and GJIC by luteal cells. In addition, progesterone secretion, Cx43 mRNA expression, and the rate of GJIC were affected by the day of the estrous cycle, cell density and/or treatment with LH and/or dbcAMP. Although it has been previously suggested that gap junctions are involved in ovarian steroidogenesis (Grazul-Bilska *et al.* 1998a, 2001, 2002), this is the first report showing a positive correlation between progesterone production, Cx43 mRNA expression, and the rate of GJIC of ovine luteal cells from several stages of the estrous cycle. Furthermore, we demonstrated that inhibition of Cx43 mRNA expression by Cx43 siRNA decreased the production of progesterone by luteal cells. This observation suggests that gap junctional Cx43 is involved in the regulation of progesterone production by luteal cells in sheep.

Primary cultures of endocrine cells have been used to investigate the role of gap junctions in regulation of cellular function in numerous studies (Oyoyo *et al.* 1997, Grazul-Bilska *et al.* 1998a, Meda 2000, Shah & Murray 2001). In addition, cell density has been previously shown to influence the ability of cells to respond to applied treatments (Pate *et al.* 1987, Larson *et al.* 1997). Most cells in our cultures expressed steroidogenic activity, as demonstrated by 3 β -HSD staining, and

Table 3 Basal Cx43 mRNA expression in luteal cells from different cell density cultures (50 – 600×10^3 cells/dish) on days 5, 10, and 15 of the estrous cycle.

Day of the estrous cycle	Cell density			
	50×10^3	100×10^3	300×10^3	600×10^3
5	$0.2 \pm 0.04^*$	$0.2 \pm 0.04^*$	$0.3 \pm 0.1^{*†}$	$0.4 \pm 0.1^{*†}$
10	$0.2 \pm 0.01^*$	$0.3 \pm 0.04^*$	$0.7 \pm 0.1^{†§}$	$0.9 \pm 0.1^{†§}$
15	$0.2 \pm 0.02^*$	$0.2 \pm 0.04^*$	$0.4 \pm 0.1^{††}$	$0.4 \pm 0.1^{††}$

Values are expressed as mean $\pm$ S.E.M. Individual basal values were obtained from each cell culture density without LH or dbcAMP-treatments.

*†Within a row, means with different superscripts differ; $P < 0.05$ for days 5 and 10, and $P < 0.1$ for day 15 of the estrous cycle. †§Within a column, means with different superscripts differ ($P < 0.001$).

Figure 3 Effects of LH and dbcAMP on Cx43 mRNA expression in luteal cells from days 5, 10, and 15 of the estrous cycle. Data are presented as a percentage of the control (no treatment, 100%). For basal Cx43 mRNA expression in control (no treatment) cultures, see Table 3. Data are combined for all cell densities within a day of the estrous cycle. Mean $\pm$ S.E.M. compared with control (no treatment; 100%) differ; * $P < 0.03$ and † $P < 0.1$.

expressed Cx43 on luteal cell borders, which indicated the presence of functional gap junctional channels.

The positive correlation between progesterone secretion and Cx43 mRNA expression, and between Cx43 mRNA expression and GJIC of luteal cells demonstrated in the present study indicates that structural and functional gap junctions are involved in the regulation of luteal steroidogenesis, and further implies that Cx43 is involved in the regulation of progesterone secretion. The decreased progesterone secretion by ovine luteal cells with siRNA-suppressed Cx43 mRNA expression confirms this observation. Furthermore, it has been demonstrated that Cx43 is involved in the regulation of the secretory function of adrenal cells (Oyoyo *et al.* 1997), and that structural and functional channels formed by Cx43 are essential in the steroidogenic processes of bovine adrenal tissue (Shah & Murray 2001). In addition, a positive correlation between increased Cx43 protein expression and enhanced insulin secretion by B-cells of the pancreas was demonstrated recently in *in vivo* and *in vitro*

Figure 4 Effects of LH and dbcAMP on the rate of GJIC of luteal cells in cultures with 50×10^3 , 100×10^3 , 300×10^3 , and 600×10^3 cells/dish from days 5, 10, and 15 of the estrous cycle. Number of cells for each density and treatment ranged from 48 to 155. Within a day of the estrous cycle and cell culture density, mean $\pm$ S.E.M. compared with control (no treatment; 100%) differ; ^{a,b,c} $P < 0.05$.

Table 4 Correlation coefficient (r) between progesterone (P4) concentration in medium (expressed as ng/ml and as pg/cell based on progesterone concentration in 1 ml medium), Cx43 mRNA expression and GJIC in luteal cells.

Overall	P4 ng/ml	P4 pg/cell	Cx43 mRNA	GJIC
P4 ng/ml	–	0.544 $P < 0.001$	0.601 $P < 0.001$	0.480 $P < 0.001$
P4 pg/cell	0.544 $P < 0.001$	–	0.220 $P < 0.003$	0.055 $P < 0.5$
Cx43 mRNA	0.601 $P < 0.001$	0.220 $P < 0.003$	–	0.532 $P < 0.001$

evaluations (Collares-Buzato *et al.* 2001, Leite *et al.* 2005). Therefore, the above observations demonstrate that both Cx43 and GJIC are involved in the regulation of hormone production in several endocrine glands including the CL.

In the present study, basal progesterone secretion was the greatest in the mid-luteal stage when the CL is completely differentiated, represented by day 10 of the estrous cycle. This observation agrees with previously published results showing a similar pattern of

Figure 5 Representative micrographs of positive Cx43 staining in cultures with 50×10^3 (A) and 300×10^3 (B) luteal cells/dish from day 5 of the estrous cycle. The morphology and staining pattern in cells with other densities and cells from days 10 and 15 of the estrous cycle were similar (data not shown). Control sections did not exhibit positive Cx43 staining (not shown). Magnification $400\times$.

Figure 6 Representative micrographs of 3 β -HSD staining in cultures with 50×10^3 (A), 100×10^3 (B), 300×10^3 (C), and 600×10^3 (D) luteal cells/dish from day 5 of the estrous cycle. Staining of luteal cells from days 10 and 15 was similar to day 5 of the estrous cycle. Insert in A–B demonstrates a lack of steroidogenic activity in control staining. Magnification 200 $\times$.

progesterone secretion *in vitro* and *in vivo* in sheep (Jablonka-Shariff *et al.* 1993, Grazul-Bilska *et al.* 1996b, 2001). We have also demonstrated that LH has greater stimulatory effects on progesterone production at the earlier rather than later stages of luteal development (day 5 versus days 10 and 15 of the estrous cycle). In addition, dbcAMP treatment increased progesterone production across all days of the estrous cycle. Responsiveness to LH and dbcAMP demonstrates that cells used in this study were functional. These stimulatory effects of LH and/or dbcAMP on progesterone production by luteal cells have also been demonstrated previously in sheep (Niswender & Nett 1994, Grazul-Bilska *et al.* 1995, 2001, Niswender *et al.* 2000).

Since it has been hypothesized that cellular interactions are involved in the regulation of progesterone secretion, several studies have been performed to determine the effects of cell–cell contact on progesterone secretion by luteal cells, but contradictory results have been reported. Pate *et al.* (1987) demonstrated that basal progesterone production and/or utilization of low- or high-density lipoproteins by bovine luteal cells, was not affected by cell density, while LH-induced progesterone production was greater in low-density cultures when compared with medium- or high-density cultures during short-term treatment. Alternatively, it has been demonstrated that bovine luteal cells without contact secreted less basal and LH-induced progesterone than those with established cellular contacts, and cellular contacts were required for maximal stimulation of progesterone synthesis by LH

(Del Vecchio *et al.* 1995a, 1995b). However, in our study, cell density did not affect LH or dbcAMP-induced progesterone secretion when evaluated on a per cell basis and the reason for discrepancies between our results and others may be due to species-specific differences. Our data have shown that progesterone production by luteal cells depends on the stage of luteal development, and LH and dbcAMP effects. However, basal- and LH- or dbcAMP-stimulated progesterone secretions do not depend on cell–cell contact *in vitro* in this study.

This and previous studies have demonstrated that, in sheep, Cx43 protein and/or mRNA expression in the CL

Figure 7 Effects of transfection of ovine luteal cells with Cx43 siRNA using targeted sequence (TS) 8 (open bars) and 24 (black bars) on Cx43 mRNA expression and progesterone production *in vitro*. Data are presented as a percentage of the control (no treatment, 100%) and expressed as mean $\pm$ s.e.m. Since day of the estrous cycle did not have any effect, data are combined for days 5 and 10. In control cultures, relative Cx43 mRNA expression was 0.7 ± 0.1 and progesterone concentration in medium was 270 ± 42 ng/ml. Mean $\pm$ s.e.m. compared with control (100%) differ; * $P < 0.02$.

were the highest during the early and mid-luteal stages of the estrous cycle, and that Cx43 protein was present on steroidogenic luteal cell borders (Grazul-Bilska *et al.* 1997a, 1997b, 1998b, 2001, Borowczyk *et al.* 2006a). In the present study, treatment with either LH or dbcAMP increased Cx43 mRNA expression during the early and mid-luteal stages of the estrous cycle. Also, Grazul-Bilska *et al.* (2000) reported that LH treatment increased Cx43 protein expression in ovine luteal tissues *in vivo*. In addition, Cx43 mRNA expression was the greatest in higher cell densities in this study. In greater cell density cultures, almost all cells remained in contact with other cells, and therefore multiple gap junctional channels were formed. These gap junctional channels are very likely formed by Cx43 since Cx43 protein was detected on luteal cell borders. This supports the previous observation that Cx43 is a major connexin in the luteal tissue. In agreement with our data, Rosenberg *et al.* (1996) demonstrated that Cx43 mRNA expression increased in parallel to cell density in two different rat hepatic cell lines. These results suggest that Cx43 may play a regulatory role in early and differentiated CL, and is likely involved in the regulation of luteal function.

In the current study, luteal cells from all stages of the estrous cycle established GJIC, and both LH and dbcAMP increased GJIC at the early and mid-luteal stages in cultures with the highest cell densities. Similarly, GJIC in confluent luteal cell cultures was affected by hormonal treatments, for example LH and dbcAMP, and was greater at the early and mid-luteal than the late-luteal stage of the estrous cycle in cows and sheep (Redmer *et al.* 1991, Grazul-Bilska *et al.* 1996a, 1996b, 1997a, 1997b, 2001). Also, in several other cell types, cAMP has been shown as a potent stimulator of GJIC (Saez *et al.* 1993). Therefore, it seems that GJIC is important for signal transduction during luteal tissue growth, differentiation, and regression. Since, during the late luteal phase, LH and dbcAMP stimulated progesterone secretion, but not GJIC and Cx43 expression, it seems that regulation of these two processes is independent during luteal regression. However, gap junctions seem to be necessary for luteolytic process, since during CL regression expression of Cx43 mRNA and protein, and GJIC are maintained at a relatively high level (this study; Grazul-Bilska *et al.* 1998b, Borowczyk *et al.* 2006a). In fact, it has been hypothesized that gap junctions facilitate transferring of luteolytic signal within luteal tissues (Niswender & Nett 1994, Grazul-Bilska *et al.* 1997a, 1997b).

In the low cell density cultures, the rate of GJIC of luteal cells and Cx43 mRNA expression were less than in the high cell density cultures, but these cells produced similar levels of progesterone. In addition, LH and dbcAMP stimulated progesterone and Cx43 mRNA expression in all cell density cultures, and stimulated GJIC in the greater cell density but not lower cell density cultures. In the low cell density cultures, fewer gap junctional channels were

formed on luteal cell borders, as observed by immunolocalization of Cx43 proteins. Therefore, with fewer gap junctions present, the increase of dye transfer in LH or dbcAMP-stimulated luteal cells in the lower cell density cultures could not be detected because the FRAP technique was not sensitive enough to detect this low level of dye transfer.

CL function is regulated by several factors including LH, which may activate second messengers including cAMP, protein kinases (PKC or PKA), or calcium, and LH has stimulatory effect on progesterone synthesis (Leung & Steele 1992, Grazul-Bilska *et al.* 1997b, 2001, Niswender *et al.* 2000, Davis & Rueda 2002). It has been clearly demonstrated that LH stimulates progesterone secretion by increasing cAMP concentration in luteal cells (Milvae *et al.* 1996). In addition, cAMP has been shown to stimulate progesterone production by luteal cells in several species (Leung & Steele 1992). Also, our study confirmed the previous observation that LH and/or dbcAMP have a stimulatory effect on progesterone production and on the rate of GJIC between luteal cells (Grazul-Bilska *et al.* 1996a, 1996b, 1997a, 1997b). However, our study demonstrated that LH has an additional function in the CL, which is the stimulation of Cx43 mRNA expression in ovine luteal cells.

Connexins are considered as proteins with the main function of forming gap junctional channels between adjacent cells (Goodenough *et al.* 1996, Kumar & Gilula 1996, Yamasaki & Naus 1996). However, this and other studies have demonstrated that connexins may play additional cellular functions independent of formation of gap junctional channels, including regulation of endocrine function (Meda *et al.* 1993, Munari-Silem & Rousset 1996, Oyoyo *et al.* 1997, Wynn *et al.* 2002) and control of cell and/or tissue growth (Yamasaki & Naus 1996, Naus 2002). Moreover, Moorby & Patel (2001) demonstrated that Cx43 had a dual function in regulating cellular growth independently of gap junction formation. In fact, Moorby & Patel (2001) have clearly shown that Cx43 acts directly on cell behavior in gap junction-independent mechanisms and has a unique role when compared with other connexins. In addition, gap junctional proteins interact with other proteins (e.g. protein kinases), which regulate gap junctional communication at several stages of the connexin 'lifecycle', including the trafficking, assembly/disassembly of gap junctional channels in the plasma membrane, connexin turnover, and the gating of gap junctional channels (Lampe & Lau 2000, Duffy *et al.* 2002, Herve *et al.* 2004). Therefore, our and other studies demonstrate that gap junctional proteins may have multiple functions.

We observed inhibition of luteal Cx43 mRNA expression by siRNA in this study, but the decrease was only about 40% of control. This indicates that the siRNA used in the present experiment did not fully suppress expression of Cx43. Progesterone secretion was also only

partially suppressed by siRNA for Cx43 in this study. Cx43 likely is not the only connexin involved in the regulation of steroidogenesis in the CL. In fact, other connexins including Cx26, Cx32, and Cx37 have been detected in luteal tissues in sheep (Grazul-Bilska *et al.* 1998b, Borowczyk *et al.* 2006a, 2006b). Cx37 protein was localized to ovine luteal cell borders, indicating that Cx37 may also be involved in the formation of gap junctions between luteal cells, and possibly in the regulation of progesterone secretion (Borowczyk *et al.* 2006b). Heterologous interactions between connexins have been well characterized indicating that different connexin composition (e.g. Cx26/Cx32 and Cx43/Cx37) may form a functional heterotypic or heteromeric channel (Stauffer 1995, Jiang & Goodenough 1996, Brink *et al.* 1997, Weber *et al.* 2004). Thus, it is very likely that Cx43 may be co-expressed or form heterotypic channels and thus share functions with other connexins in the CL. In organs, such as the liver and pancreas, Cx26 and Cx32 are highly expressed and the integrity of both connexins is necessary for normal glandular secretory function. Interestingly, in Cx32-knockout mice, glandular secretion was significantly reduced; however, Cx26 was still expressed and maintained liver secretory functions (Walcott *et al.* 2002). This indicates that in the absence of one connexin, some other connexin(s) is(are) sufficient to maintain normal organ function. Therefore, further studies using a combination of siRNA cocktails against several connexins should be performed in luteal cell cultures, in order to clearly demonstrate the role of connexins in the regulation of the CL function. In fact, regulation of luteal function is a complex process (Niswender & Nett 1994, Milvae *et al.* 1996, Pate 1996, Niswender *et al.* 2000, Stouffer 2006); therefore, gap junctional proteins seem to be only a part of the complex mechanism contributing to normal function of the CL.

Our results did not fully support our hypothesis that cell–cell contact affects basal or induced progesterone secretion by luteal cells *in vitro*. However, we have demonstrated a positive relationship between progesterone production, Cx43 mRNA expression, and the rate of GJIC in ovine luteal cells. Therefore, our data suggest that Cx43 is involved in the regulation of progesterone production by ovine luteal cells. However, future studies should be undertaken using siRNA cocktail for connexin genes to characterize the role of gap junctions and specific connexins in the regulation of progesterone secretion and expression of enzymes controlling steroidogenesis within the CL.

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