Involvement of connexin 43 in meiotic maturation of bovine oocytes

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In ovarian follicles, cumulus cells provide the oocyte with small molecules that permit growth and control maturation. These nutrients reach the germinal cell through gap junction channels, which are present between the cumulus cells and the oocyte, and between the cumulus cells. In this study the involvement of intercellular communication mediated by gap junction channels on oocyte maturation of *in vitro* cultured bovine cumulus–oocyte complexes (COCs) was investigated. The stages of oocyte maturation were determined by Hoechst 33342 staining, which showed that 90% of COCs placed in the maturation medium for 24 h progress to the metaphase II stage. Bovine COC gap junction communication was disrupted initially using *n*-alkanols, which inhibit any passage through gap junctions. In the presence of 1-heptanol (3 mmol l⁻¹) or octanol (3.0 mmol l⁻¹ and 0.3 mmol l⁻¹), only 29% of the COCs reached metaphase II. Removal of the uncoupling agent was associated with restoration of oocyte maturation, indicating that treatment with *n*-alkanols was neither

cytotoxic nor irreversible. Concentrations of connexin 43 (Cx43), the major gap junction protein expressed in the COCs, were decreased specifically using a recombinant adenovirus expressing the antisense Cx43 cDNA (Ad-asCx43). The efficacy of adenoviral infection was > 95% in cumulus cells evaluated after infection with recombinant adenoviruses expressing the green fluorescence protein. RT-PCR performed on total RNA isolated from Ad-asCx43-infected COCs showed that the rat Cx43 cDNA was transcribed. Western blot analysis revealed a three-fold decrease in Cx43 expression in COCs expressing the antisense RNA for Cx43. Injection of cumulus cells with Lucifer yellow demonstrated further that the resulting lower amount of Cx43 in infected COCs is associated with a two-fold decrease in the extent of coupling between cumulus cells. In addition, oocyte maturation was decreased by 50% in the infected COC cultures. These results indicate that Cx43-mediated communication between cumulus cells plays a crucial role in maturation of bovine oocytes.

Introduction

In ovarian follicles, cumulus cells play an important role in oocyte growth and differentiation by providing the germinal cell with nutrients and by controlling both nuclear and cytoplasmic maturation when oocytes are selected for ovulation (Eppig, 1996). In response to gonadotrophin *in vivo*, the fully grown immature oocytes resume maturation and undergo germinal vesicle breakdown (GVBD), progressing from prophase of the first meiotic division to metaphase II (Dekel and Beers, 1980). Several layers of tightly adherent cumulus cells surround the immature mammalian oocytes. There is a complex interplay between oocytes and cumulus cells during folliculogenesis (Buccione *et al.*, 1990; Gougeon, 1996) that is mediated by growth factors and hormones. Cumulus cells communicate through an intricate network of transmembrane channels called gap junctions (Grazul-Bilska *et al.*, 1997); the innermost layer of

cumulus cells extends cytoplasmic processes through the zona pellucida, a thick extracellular matrix, forming gap junctions with the oocyte (White and Paul, 1999). These junctional complexes, composed of proteins belonging to the connexin family (Bruzzone *et al.*, 1996; Kumar and Gilula, 1996), mediate the exchange of ions and metabolites (up to 1 kDa) between neighbouring cells (Goodenough *et al.*, 1996).

Simon *et al.* (1997) demonstrated that connexin 37 (Cx37) is present in gap junctions between the oocyte and the cumulus cells, and that Cx37-deficient mice lack mature follicles, fail to ovulate and develop inappropriate corpora lutea. In Cx37 knockout mice, cumulus–oocyte cell coupling is ablated, although cumulus–cumulus cell coupling persists (White and Paul, 1999). The junctions joining the cumulus cells are formed primarily of Cx43 (Beyer *et al.*, 1989) and during *in vitro* culture, transfer of Lucifer yellow between cumulus cells still occurs after a 24 h period (Sutovsky *et al.*, 1993).

Oocytes lack many transport systems for nutrient uptake (nucleotides, amino acids and sugars) and, in rodent

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species, gap junction transfer of nutrients from cumulus cells to the oocyte is required for oocyte growth (Eppig, 1996), whereas transfer of cAMP inhibits oocyte maturation (Dekel, 1988; Granot and Dekel, 1997). In cows, the effects of cAMP on oocyte cell cycle regulation are not established as clearly as in mice; meiotic inhibition in bovine oocytes is the result of co-operation among cumulus, granulosa and theca cells (Richard and Sirard, 1996a,b; Richard *et al.*, 1997). The function of cumulus cells may be more complex than the simple transmission of cAMP inhibition signals. These cells may also transmit a positive signal leading to meiotic resumption, which can reverse the inhibitory signal by stimulating the cumulus to produce a GVBD-inducing factor (Sirard and Bilodeau, 1990a,b). In pig ovaries, Cx43 was localized to the granulosa cells of the developing follicle and it was demonstrated that gonadotrophin stimulation enhances the intensity of immunoreactive Cx43 (Lenhart *et al.*, 1998). Metabolic coupling experiments have shown that the coupling between cumulus cells and the oocyte persists at the time of meiotic resumption by oocytes (Heller and Schultz, 1980; Eppig, 1982; Bornslaeger and Schultz, 1985; Larsen *et al.*, 1986; de Loos *et al.*, 1991; Laurincik *et al.*, 1992). Therefore, the integrity of the gap junctional network could be necessary for induction of meiotic maturation of oocytes.

In the present study, the physiological role played by the gap junction network in meiotic maturation of oocytes *in vitro* was investigated. All gap junction communication was first blocked by exposing bovine COCs to several alkanols known as gap junction uncouplers (Christ, 1995; Largo *et al.*, 1997; Chanson *et al.*, 1998; Venance *et al.*, 1998). In a second series of experiments, Cx43 expression was disrupted specifically by infecting the COCs with a recombinant adenovirus expressing a Cx43 antisense RNA.

Materials and Methods

Recovery of the cumulus–oocyte complexes

Cow ovaries were obtained from the local abattoir. Immediately after collection, the ovaries were placed into sterile PBS supplemented with 50 µg gentamycin ml⁻¹ and maintained at 39°C in a waterbath. The ovaries were transported to the laboratory within 1–2 h in an isotherm box maintained at about 35–39°C and washed in sterile PBS with gentamycin. The follicular fluids were aspirated gently from small antral follicles (2–8 mm in diameter) with a needle (19G) and a syringe (5 ml). The sedimented COCs were transferred into 2 ml Hepes-buffered medium 199 (M199-Hepes; Gibco BRL, Basel), supplemented with 10% synthetic serum substitute (SSS; Irvine Scientific, San Francisco, CA) and 50 µg gentamicin ml⁻¹, and adjusted to a final osmolarity of 290 ± 2 mOsmol l⁻¹. All the COCs were recovered under a stereomicroscope and rinsed several times with M199-Hepes. The COCs were pooled in two groups (A and B) following the morphological criteria of Blondin and Sirard (1995) and Chanson *et al.* (2001). COCs from group B were discarded.

Maturation medium and n-alkanols

Oocyte meiotic maturation was carried out in a bicarbonated M199 medium (M199-HCO₃; Gibco BRL) adjusted to a final osmolarity of 290 ± 2 mOsmol l⁻¹ and supplemented with 10% SSS containing 84% human serum albumin, 16% α- and β-globulins, < 1% γ-globulins (Weathersbee *et al.*, 1995; Tucker *et al.*, 1996), 1 iu recombinant FSH ml⁻¹ (Gonal F; Serono, Geneva), 1 iu LH ml⁻¹ (LHADI; Serono) and 1 µg oestradiol ml⁻¹ (Sigma Chemical Co., St Louis, MO), in the presence or absence of standard concentrations of *n*-alkanols (3 mmol 1-heptanol l⁻¹; 3.0 or 0.3 mmol octanol l⁻¹) (Christ, 1995; Largo *et al.*, 1997; Chanson *et al.*, 1998; Venance *et al.*, 1998). The COCs (20 per well) were placed in 0.5 ml medium. *In vitro* maturation was carried out by culturing the COCs for 22–24 h under standard incubation conditions (39°C, 5% CO₂ and 95% air) in a humidified incubator (BB 16; Heraeus, Hanau). Each experimental replicate consisted of approximately 80 COCs retrieved from about 20 ovaries. The COCs were incubated in the culture medium with and without 3 mmol 1-heptanol l⁻¹ to measure the reversibility of the effects of 1-heptanol. After 24 h of culture, about 50% of the COCs of each group were processed to analyse their stage of maturation. The remaining 50% of each group were washed several times in the culture medium to remove 1-heptanol. The COCs were cultured for 24 h in the control medium. The index of maturation used in the present study was GVBD; to determine the maturational stage of the oocytes after 24 ± 1 h or 48 ± 1 h of culture, the oocytes were denuded in the presence of 80 iu hyaluronidase ml⁻¹ (vortexed for 10 min). The DNA of the germinal cells was stained with Hoechst 33342 (10 µg ml⁻¹ in PBS) and visualized on an inverted fluorescence microscope. The presence of two fluorescent spots was indicative of metaphase II stage; when only one spot was detected the oocytes were considered to be in the germinal vesicle stage.

Generation of recombinant adenoviruses

Recombinant adenoviruses comprising the complete cDNA of rat Cx43 (Beyer *et al.*, 1987) in the antisense orientation were constructed and fused (Ad-asCx43-GFP) or not (Ad-asCx43) to the green fluorescent protein (GFP) cDNA to inhibit Cx43 expression. Adenovirus that expresses GFP only was used as a control. The GFP coding region was ligated into pCIneo (pCIneo-GFP) to generate the Ad-GFP virus. The cassette of pCIneo-GFP encompassing the cytomegalovirus (CMV) promoter, the artificial intron of pCIneo, the coding region of GFP and the poly A addition signal of pCIneo was inserted into pXC15-18 (Schaack *et al.*, 1995) to produce the adenoviral transfer vector pXC-GFP. Ad-GFP was generated by homologous recombination in 293 cells after co-transfection by the calcium phosphate procedure of pJM17 (Hitt *et al.*, 1998) and pXC-eGFP. Viruses collected at day 10 after transfection were plaque-purified three times on HR911 cells (IntroGene, Leiden). Plaque assays were performed in six-well dishes with 30%

(v/v) confluent HR911 cells. Cells were infected with 400 μ l virus suspension in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 250 U penicillin ml^{-1} (Gibco BRL) and 250 μ g streptomycin ml^{-1} (Gibco BRL) for 1 h, at which time the inoculum was replaced by 2.5 ml per well of freshly prepared overlay equilibrated at 40°C. The overlay contained 2 $\times$ minimal essential medium (a five-fold dilution of 10 $\times$ MEM; Life Technologies, Paisley), 20% FCS and 2.5% (w/v) Seaplaque agarose (FMC; Bioconcept, Allschwill). After the agarose solidified, the dishes were returned to the CO₂ incubator and the plaques were scored at day 5 after infection. Viruses from three independent plaques were analysed by restriction enzyme digestion of viral DNA purified from 293 cells by the Hirt procedure (Hirt, 1967). Large stock viruses were purified by two rounds of caesium chloride centrifugation. The virus band (1.5 ml) was collected and dialysed at 4°C against 10 mmol Hepes l^{-1} , pH 8.0, 150 mmol NaCl l^{-1} in a Slide-A-Lyzer (0.5–3.0 ml capacity), gamma-irradiated 10 K dialysis cassette (Pierce, Rockford, IL). The adenovirus is an E1-E3 deleted, replication defective, recombinant human adenovirus type 5 allowing expression of GFP or the Cx43 antisense RNA or both under the control of the strong immediate early CMV promoter. COCs were incubated in the maturation medium with recombinant adenoviruses at approximately 50–100 virus particles per COC for 24 h.

RT-PCR amplification

COCs ($n = 35$) frozen in liquid nitrogen were homogenized in 4 mol guanidium isothiocyanate buffer l^{-1} , containing 25 mmol sodium citrate l^{-1} and 100 mmol β -mercaptoethanol l^{-1} . Total RNA from COCs, either infected or uninfected, was extracted by the acid guanidium–isothiocyanate method (Chomczynski and Sacchi, 1987) and yields were evaluated by measuring absorbance at 260 nm. Total RNA was treated for 30 min in the presence of 20 U DNase I (Pharmacia, Dubendorf). Aliquots (1 μ l) of DNase-treated RNA were reverse-transcribed as described by Haefliger *et al.* (2000).

The reverse-transcribed products were used for PCR reaction using Taq DNA polymerase (Gibco BRL) in the presence of 20 ng sense primer and 20 ng antisense primer (MWG-Biotech, München). The sense primer 5' TCTAC-GTCTTCTCAAAGGCG 3' sequence is located in the rat Cx43 sequence; the other primer 5' AGTCAGTGCTTCT-GACACA 3' originated in front of the intron sequence of the viral DNA. These primers amplified a fragment of 657 bp. As positive controls, samples of viral DNA prepared by overnight digestion of purified virus particles were subjected to PCR amplification. The amplified DNA was visualized after ethidium bromide staining on an agarose gel.

Western blot analysis

Infected COCs or control COCs were homogenized in 100 mmol Tris–HCl l^{-1} , pH 7.4, supplemented with

20 mmol EDTA l^{-1} , 1 μ g pepstatin A ml^{-1} , 1 μ g antipain ml^{-1} (all Merck, Dietikon), 1 mmol benzamide l^{-1} , 40 kallikrein inactivator units (KIU) aprotinin ml^{-1} (Bayer, Zurich), 2 mmol phenylmethylsulphonyl fluoride l^{-1} (PMSF; Sigma Chemical Co.) and 1 mmol diisopropyl fluorophosphate l^{-1} (DFP; Aldrich Chemical Co., Milwaukee, WI). The homogenates were passed through a needle to shear the DNA and centrifuged at 3000 *g* for 10 min to pellet intact cells. Supernatants were collected and centrifuged for 60 min at 100 000 *g* at 4°C. The crude membrane preparation was pelleted and resuspended in a solubilization buffer containing 62.5 mmol Tris l^{-1} , pH 8.0, 5% (w/v) SDS and 10 mmol EDTA l^{-1} . Samples of crude membrane preparation were fractionated by electrophoresis in a 12.5% (w/v) polyacrylamide gel and immunoblotted onto Immobilon PVDF membranes (Millipore Co., Volketswil). The membrane was stained with Ponceau S solution (Sigma, Steinheim) and incubated for 4 h with a monoclonal antibody against Cx43 (Zymed laboratories Inc., San Francisco, CA) diluted 1:10 000 in blocking buffer. For quantification, the lanes were cut longitudinally in two parts. One part was incubated for 4 h with a monoclonal antibody against Cx43 (Zymed laboratories Inc.) diluted 1:10 000 in blocking buffer (Haefliger *et al.*, 1997, 1999) and the other part was incubated with a monoclonal antibody against α -tubulin (T5168; Sigma) diluted 1:10 000 in blocking buffer. After repeated rinsing in PBS and PBS + 0.1% (v/v) Tween 20, immunoblots were incubated overnight at 4°C with an anti-mouse IgG antibody coupled with alkaline phosphatase (Dako Diagnostic AG, Zug) diluted 1:5000. The bands were developed with BCIP-NBT method (AP development reagent; BioRad Laboratories, Glattburg).

Lucifer yellow microinjection

Intercellular communication between the cumulus complex cells was assessed by Lucifer yellow microinjection. The oocyte of the COCs was held with a micropipette and an individual cell was impaled within each COC with a glass microelectrode filled with a 4% (v/v) Lucifer yellow CH (Sigma Chemical Co.) solution in 3 mmol lithium chloride l^{-1} buffered to pH 7.2 with 10 mmol Hepes l^{-1} . The tracer was pressure-injected into the cell for 30 s. The injections were carried out on an epifluorescence-equipped inverted microscope (IMT2; Olympus, Zürich) with Hofman contrast and a heated stage at 37°C. Colour slides of the microinjected COCs were projected and the extent of dye coupling was evaluated by counting the number of Lucifer yellow-labelled cells within each COC.

Statistical analysis

Densitometric analysis of immunolabelled proteins (western blot analysis) was performed using a Molecular Dynamics scanner (Sunnyvale), which integrates areas and corrects for background. Signals for Cx43 were related to the corresponding tubulin signals. Relative protein concentrations calculated from western blot analysis and the extent

of dye diffusion were compared using superANOVA and Scheffe's test. Data are expressed as mean $\pm$ SEM. Statistical significance was defined at values of $P < 0.05$ and $P < 0.01$. The statistical analysis of oocyte meiotic maturation was performed using a contingency table (StatView for Macintosh).

Results

Effects of n-alkanols on meiotic maturation of oocytes

Freshly collected oocytes were immature at the beginning of the culture period, as indicated by Hoechst staining of denuded oocytes, which demonstrated that all oocytes were in the germinal vesicle stage. After 24 h in the maturation medium, 85% (28 of 33) of the oocytes present in COCs resumed meiosis and were recovered in the metaphase II stage. In contrast, only 53% (49 of 92) of the oocytes matured when denuded before *in vitro* culture, indicating that cumulus cells play a role in oocyte maturation.

Two gap junction uncoupling agents (1-heptanol and octanol) were added to the maturation medium for 24 h to investigate the involvement of gap junctions in meiotic maturation of oocytes. Cumulus cell expansion and morphology of oocytes cultured in the medium with heptanol were similar to those of control COCs matured without heptanol or octanol. In the presence of 3.0 or 0.3 mmol octanol l^{-1} , 77.6% of the oocytes were blocked at the germinal vesicle stage ($n = 74$ oocytes in total), whereas 98.6% of the control oocytes ($n = 76$ oocytes in total) resumed meiosis in maturation medium (Fig. 1a). When 3 mmol 1-heptanol l^{-1} was added to the culture medium ($n = 115$ oocytes in total) only 29% of the oocytes matured to the metaphase II stage, whereas 50% of the oocytes remained in the germinal vesicle stage and 21% matured to an intermediate stage (Fig. 1b). In the same experiment, 90% of control oocytes ($n = 93$ oocytes in total) were recovered in metaphase II stage, 4% remained in the germinal vesicle stage and 6% were recovered at an intermediate stage of maturation (Fig. 1b). The reversibility of 1-heptanol inhibition of maturation was studied further. After 24 h in the presence of 1-heptanol, only 28% of treated oocytes ($n = 79$ oocytes in total) resumed meiosis, whereas 88% of control oocytes ($n = 74$ oocytes in total) were recovered in metaphase II stage (Fig. 1c). Half of the oocytes incubated initially for 24 h in the presence of heptanol were incubated for a further 24 h in the absence of 1-heptanol. Removal of the uncoupling agent was associated with restoration of meiotic maturation of oocytes, as 63% of the oocytes ($n = 86$ oocytes in total) resumed meiosis, whereas 78% of control oocytes ($n = 60$ oocytes in total), which had not been incubated in the presence of heptanol, had resumed meiosis at 48 h (Fig. 1c).

Effects of a decrease in Cx43 in cumulus cells on meiotic maturation of oocytes

RT-PCR on RNA extracted from COCs infected during 24 h with Ad-asCx43 (Fig. 2a) and Ad-asCx43-GFP (data

not shown) demonstrated that antisense Cx43 RNA was transcribed 24 h after infection. The efficacy of infection was $> 95\%$, as evaluated by measuring the number of GFP fluorescent-positive cells in cumulus cells using Ad-asCx43-GFP or Ad-GFP (Fig. 2b).

A kinetic study after adenovirus infection showed that the antisense Cx43 RNA was already detectable in COCs at 2 h after infection and that maximum expression was achieved 4 h after infection (Fig. 2c) and remained stable during the next 24 h of infection. Western blot analysis of proteins extracted from COCs was performed to determine whether the presence of an antisense Cx43 RNA affected the expression of Cx43 (Fig. 2d). Total proteins extracted from heart and aorta were used as positive controls. The extracts from aorta contained one major immunoreactive band (43 kDa), whereas the heart Cx43 was immunodetectable as three bands of 43–45 kDa (Haeffliger *et al.*, 1999). A reduction of Cx43 content in COCs was already detectable 6 h after infection (Fig. 2d). Quantitative evaluation of the Cx43 expressed in COCs was performed 24 h after infection (Fig. 3). The nylon membrane lanes were cut longitudinally in two parts for this purpose. One part was incubated with Cx43 antibodies and the other part was incubated with tubulin antibodies. Statistical analysis showed that Cx43 content was significantly decreased (Fig. 3) in COCs infected for 24 h with Ad-asCx43 ($n = 4$; $P < 0.01$) compared with tubulin signals. Cumulus cell coupling was studied by Lucifer yellow injections at 24 h after infection to determine whether a decrease in Cx43 was associated with a decrease in cell–cell communication. One cumulus cell in each COC, located peripherally, was injected with Lucifer yellow; the coupling between cumulus cells infected with Ad-asCx43 was reduced by 66% compared with cumulus cells of uninfected COCs (Fig. 4). No diffusion of Lucifer yellow was detected between the injected cumulus cell and the oocyte, and addition of heptanol to COCs blocked all transfer of Lucifer yellow (data not shown).

After 24 h of culture, 91% of oocytes ($n = 67$) from control COCs had matured to the metaphase II stage. Furthermore, 89% ($n = 96$) of the oocytes from COCs infected with the Ad-GFP were recovered in the metaphase II stage, indicating that viral infection had no cytotoxic effect on meiotic maturation of oocytes. In contrast, only 48% ($n = 94$ oocytes in total) and 51% ($n = 93$ oocytes in total) of oocytes present in COCs infected with either Ad-asCx43 or Ad-asCx43-GFP, respectively, underwent GVBD (Fig. 5).

Discussion

A semi-defined medium for *in vitro* maturation of immature bovine COCs has been developed to study the importance of gap junctions in meiotic maturation of bovine oocytes (Weathersbee *et al.*, 1995; Tucker *et al.*, 1996; Chanson *et al.*, 2001). Bovine oocytes removed from small antral follicles were shown to have full meiotic competence after

Fig. 1. Effect of *n*-alkanols on *in vitro* maturation of bovine oocytes. (a) Meiotic maturation of cumulus–oocyte complexes (COCs) incubated for 24 h in maturation medium in the presence of octanol (0.3 mmol l⁻¹). A total of 77.6% of the treated oocytes (□; *n* = 74 in total) were blocked at the germinal vesicle stage, whereas only 1.4% of control COCs (■; *n* = 76 in total) did not mature and 21% matured to an intermediate or intermediate stage (anaphase I or metaphase I, data not shown). Data are expressed as mean ± SEM (*n* = 3 independent experiments). (b) In the presence of 3 mmol 1-heptanol l⁻¹ in the culture medium (□; *n* = 115 in total) only 29% of the treated oocytes matured to the metaphase II stage, whereas 50% of the oocytes remained in the germinal vesicle stage and 21% matured to an intermediate stage (data not shown). Ninety per cent of control COCs (■; *n* = 93 in total) were recovered in metaphase II stage, 4% remained in the germinal vesicle stage and 6% were recovered at an intermediate stage of maturation. Data are expressed as mean ± SEM (*n* = 5 independent experiments). (c) The reversibility of the inhibitory effect of 1-heptanol (3 mmol l⁻¹) on maturation was studied. After 24 h in the presence of 1-heptanol, only 28% (□; *n* = 79 in total) of treated oocytes were in metaphase II, whereas 88% of oocytes of control COCs had resumed meiosis (■; *n* = 74 in total). Half of the COCs incubated for 24 h in the presence of heptanol were incubated for a further 24 h in the absence of 1-heptanol. In these conditions, 63% (▨; *n* = 86 in total) of treated oocytes were recovered in the metaphase II stage, whereas 78% of oocytes of control COCs (▨; *n* = 60 in total) which had never been incubated in the presence of heptanol, had resumed meiosis after 48 h. Data are expressed as mean ± SEM (*n* = 4 independent experiments). ****P* < 0.001 and ***P* < 0.01.

Fig. 2. Ad-asCx43-transcribed connexin 43 (Cx43) antisense RNA in bovine cumulus-oocyte complexes (COCs). (a) Schematic diagram depicting the RT-PCR strategy with the localization of the primers A and B. RT-PCR was performed using two oligonucleotides flanking the intron of 132 bp to differentiate between the viral DNA and the RNA transcribed from the viral DNA. Total RNA was isolated from Ad-asCx43-infected COCs. PCR or RT-PCR products were size-fractionated on an agarose gel and stained with ethidium bromide. Lane 1: PCR amplified product of viral Ad-asCx43 genomic DNA extracted from Ad-asCx43; a single 789 bp product was amplified. Lane 2: RT-PCR product amplified from RNA extracted from bovine COCs infected with Ad-asCx43; a single 657 bp product was amplified. The sequence of 132 bp corresponding to the intron size was removed. Lane 3: PCR and RT-PCR products together to visualize the 132 bp difference. No band was amplified from RNA extracted from non-infected COCs (data not shown). (b) Schematic diagram depicting the construct allowing for the expression of the green fluorescent protein (GFP) and the antisense Cx43. After 24 h of infection, the efficacy of infection was > 95% as evaluated by GFP fluorescence in cumulus cells using Ad-asCx43-GFP or Ad-GFP. (c) Reverse-transcribed RNA extracted from non-infected COCs (time = 0 h) or COCs infected with Ad-asCx43 at different times after infection was amplified by PCR using specific primers. A single 657 bp product was amplified. Antisense Cx43 transcripts were detected after 2 h of infection. This product was not observed in samples that were not reverse transcribed before PCR (data not shown). PCR amplification of genomic viral DNA resulted in the amplification of a 789 bp product because of the presence of a 132 bp intron. (d) Western blot analysis revealed that, compared with controls (time = 0 h), Cx43 was decreased in COCs infected with the adenovirus expressing the Cx43 cDNA in the antisense orientation (Ad-asCx43) after 6 h of infection. Samples of heart and aorta were used as positive controls. Cx43 was detected as three bands of 43–45 kDa in heart and one band in aorta. Lanes of heart and aorta samples were loaded with 40 µg total protein. Lanes of COC samples were loaded with 50 µg total protein.

24 h in this defined medium, as they could be fertilized efficiently with spermatozoa (Chanson *et al.*, 2001).

Long chain alcohols (1-heptanol or octanol), which block gap junction channels in a rapid manner (Chanson *et al.*, 1989), were used to interfere with intercellular communication. Heptanol reduces gap junctional coupling between cumulus cells and oocytes (Fagbohun and Downs, 1991) and can be used to inhibit the function of cumulus cells during oocyte maturation without having to remove them. In the present study, the involvement of intercellular communication mediated by gap junction channels in oocyte maturation of *in vitro* cultured bovine COCs was

investigated. In the presence of these alkanols, only 29% of the COCs reached metaphase II and removal of the uncoupling agent was associated with restoration of oocyte maturation. These data indicate that gap junction-mediated communication between cumulus cells plays a crucial role in maturation of bovine oocytes. The same uncoupling agents have been used in *Xenopus laevis* to examine the role of follicle cell-oocyte gap junction coupling (Patino and Purkiss, 1993); these data indicate that gap junctional coupling between follicle cells and oocytes is required for maturational steroid signalling in amphibian ovaries. Using heptanol, Coskun and Lin (1994) showed that transforming

Fig. 3. Decreased connexin 43 (Cx43) content in bovine cumulus–oocyte complexes (COCs) infected with Ad-asCx43. (a) Western blot analysis of Cx43 revealed that the content of this protein (43 kDa band) was lower in the COCs infected with the adenovirus expressing the Cx43 cDNA in the antisense orientation (Ad-asCx43) than in COCs infected with Ad-GFP (green fluorescence protein). This difference was not observed with α -tubulin (50 kDa), indicating that the same amount of total proteins was loaded (50 μ g). (b) Quantitative evaluation of four immunoblots revealed that Cx43 content was reduced by 65% in samples of COCs infected with Ad-asCx43. **Significantly different from control ($P < 0.01$).

growth factor α (TGF- α) induced signalling from cumulus cells to oocytes via gap junctions. Recently, using the same blocking agent, Mori *et al.* (2000) demonstrated that gap junctions play an important role in regulating the cytoplasmic factors responsible for the removal of sperm nuclear envelopes and glutathione inflow from cumulus cells. Downs (1995) demonstrated further that gap junctions are involved in the mediation of both stimulatory and inhibitory signals from the cumulus cells, due to the

Fig. 4. Extent of dye coupling in bovine cumulus–oocyte complexes (COCs) expressing antisense connexin 43 (Cx43) RNA. Fluorescent view of (a) a control COC or (b) a COC infected with Ad-asCx43 after injection of Lucifer yellow at 24 h after infection. (c) The extent of dye coupling within cumulus cells indicates a 65% reduction (** $P < 0.01$) in the number of Lucifer yellow-labelled cells within each COC infected with Ad-asCx43. Cell-to-cell coupling between Ad-asCx43-infected cells was significantly reduced (15 ± 2) compared with control uninfected cells (44 ± 2). Values of the extent of dye diffusion are mean $\pm$ SEM ($n = 27$ for control and $n = 21$ for infected COCs); results of seven independent experiments.

influence of glucose and ATP concentrations within mouse COCs.

de Loos *et al.* (1994) proposed that, in bovine oocytes, mural granulosa cells generate a meiosis-arresting substance

Fig. 5. Decreased maturation of bovine cumulus–oocyte complexes (COCs) infected with Ad-asCx43. The incidence of germinal vesicle breakdown of oocytes infected or not infected with adenoviruses was measured. After 24 h in culture, 91% of oocytes from control COCs matured to the metaphase II stage (■; $n = 67$ in total). In contrast, only 48% (▨; $n = 94$ in total) and 51% (▤; $n = 93$ in total) of oocytes present in COCs infected with Ad-asCx43 (adenovirus and antisense connexin 43) or Ad-asCx43-GFP (green fluorescent protein), respectively, underwent germinal vesicle breakdown. Furthermore, 89% (□; $n = 96$ in total) of the oocytes from COCs infected with Ad-GFP were recovered in the metaphase II stage, indicating that the virus alone has no cytotoxic effect on meiotic maturation of oocytes. Only one oocyte in each condition tested was found at an intermediate stage (data not shown). Data are mean $\pm$ SEM ($n = 8$ experiments). *** $P < 0.001$.

and transport it to the oocyte through gap junctions. Completion of meiosis can be induced by separating oocytes from cumulus cells (Edwards, 1965; Racowsky and Baldwin, 1989). In rodents, gap junctional communication could maintain oocyte arrest in the first meiotic prophase by delivering inhibitory signals, such as cAMP, from the cumulus cells (Sandberg *et al.*, 1992; Chesnel *et al.*, 1994). These studies indicate that gap junction communication maintains oocytes that are arrested in the first meiotic prophase. However, if this model is correct, competent oocytes lacking junctional communication with cumulus cells would be expected to undergo premature meiotic resumption. Fagbohun and Downs (1991) demonstrated that gap junctional coupling between cumulus cells and oocytes is necessary to mediate the actions of concanavalin A and FSH, which induce meiotic resumption in mice; other studies have shown that meiotic resumption precedes loss of gap junctional communication. Metabolic coupling assays performed in several different species have shown that GVBD occurs before any detectable decrease in the transport of small molecules (Schultz *et al.*, 1983; Motlik *et al.*, 1986; Hyttel, 1987; Eppig and Downs, 1988; Mattioli *et al.*, 1988; de Loos *et al.*, 1991; Laurincik *et al.*, 1992), thereby indicating that junctions between COCs remain functional until metaphase II.

The junctions coupling the cumulus cells with the oocytes are composed partly of Cx37 (Simon *et al.*, 1997); female mice lacking Cx37 are infertile because ovulation does not occur (Simon *et al.*, 1997). Only a small percentage of oocytes mature sufficiently to resume meiosis. As oocytes in female mice lacking Cx37 never acquire meiotic competence, these follicles *in vivo* do not permit us to test this model (Goodenough *et al.*, 1999). These *in vivo* data indicate that growth-promoting signals from follicular cells, necessary for oocyte meiotic maturation, permeate through gap junctions (White and Paul, 1999).

Juneja *et al.* (1999) showed that the gonads of fetal and neonatal mice lacking Cx43 are unusually small owing to a deficiency of germ cells. In addition, postnatal folliculogenesis is impaired in ovaries lacking Cx43 (Juneja *et al.*, 1999). These results imply that Cx43 is required for both germline development and the early stages of folliculogenesis in the ovaries. In cows, Cx43 is present only in granulosa cells and on the borders between granulosa cells and oocytes (Johnson *et al.*, 1999) and concentrations increase in healthy developing antral follicles. Cx32 is present only between granulosa cells of atretic and small antral follicles, whereas Cx26 is expressed in the oocytes of primordial and primary or secondary follicles, and in the granulosa of healthy antral follicles (Johnson *et al.*, 1999). In bovine ovaries, Cx37 is localized in bovine ovarian follicles and is present predominantly at preantral stages (Nuttinck *et al.*, 2000). Cx37 is detected in both oocyte and granulosa cell compartments. Cx37 expression decreases significantly at the onset of antral cavity formation, whereas Cx43 is expressed weakly in preantral follicles. These data indicate that Cx37 and Cx43 expression patterns are regulated differentially throughout folliculogenesis. This complex pattern of gap junction expression indicates that gap junctional coupling plays an indispensable role in regulating development and regression of follicles and may reflect specific physiological roles for each connexin.

An adenovirus gene transfer approach was used to decrease Cx43 expression specifically in bovine cumulus cells to study the involvement of Cx43 further. By reducing the expression of Cx43, as demonstrated by western blot analysis and injections of Lucifer yellow, the incidence of maturation was decreased by 50%, demonstrating that specific cellular communications mediated by Cx43 are critical for meiotic maturation of oocytes. The disruption of cell-to-cell communication between the cumulus cells could participate in triggering the resumption of meiosis by allowing the synergic effect of coupled cumulus cells to signal, via gap junctions, a diffusible factor necessary to elicit proper meiotic maturation of oocytes. The nature of these molecules is still unknown and the use of adenoviruses to decrease Cx43 expression between the cumulus cells may be useful to characterize those involved in meiotic resumption. Geshi *et al.* (2000) demonstrated that sodium pyruvate promotes nuclear maturation in bovine cumulus-denuded oocytes and that a continuous presence of cumulus cells during oocyte maturation is important for

subsequent development of zygotes to the blastocyst stage in cattle. These findings indicate that cumulus cells may metabolize glucose which can be passed to the oocyte, presumably through gap junctions.

In conclusion, the different approaches used in the present study indicate that gap junction-mediated communication between cumulus cells plays a crucial role in maturation of bovine oocytes. A novel strategy was used to inhibit Cx43 expression specifically and decreased Cx43 protein content resulted in significantly lower gap junction communication, as observed by Lucifer yellow staining. The present study is the first in which the major role played by Cx43 in gap junction communication in oocyte maturation has been demonstrated; this connexin appears to be largely responsible for proper gap junction communication and adequate Cx43 expression is important for maturation of bovine oocytes. These data indicate that intercellular communication mediated by gap junctions between cumulus cells is required for proper meiotic maturation of oocytes and that Cx43 plays an important role in this process.

J-A. Haefliger is supported by a career award from the Max Cloëtta Foundation. This work was supported by a grant from the Swiss National Science Foundation (31-56689.99 to J-A. Haefliger) and the Placide Nicod and Octav Botnar Foundations. The authors would like to thank P. Francfort, Veterinarian, and J-F. Deschenaux for their help during collection of the ovaries at the slaughterhouse. The recombinant LH and FSH were kindly provided by Sero (Geneva).

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Received 24 January 2001.

First decision 16 March 2001.

Final version received 8 June 2001.

Accepted 19 June 2001.